IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: James P. Elia	)	
	)	
SERIAL NO.: 09/064,000	)	EXAMINER: Nicholas D. Lucchesi
	)	
FILED: April 21, 1998	)	
	)	GROUP ART UNIT: 3732
FOR: METHOD AND APPARATUS FOR INSTALLATION OF DENTAL IMPLANT	)))	

DECLARATION OF C. GENE WHEELER, M.D., F.A.C.S.

I, C. Gene Wheeler, declare as follows:

1. I reside at 6342 E. Hillcrest Boulevard, Scottsdale, Arizona 85251.

2. My Curriculum Vitae is attached hereto as Exhibit A.

3. I have read and understood the disclosures at column 14, lines 4-61 and column 21, lines 1-26 of United States Patent Number 5,397,235 (hereinafter " '235 patent") entitled "Method for Installation of Dental Implant," and granted to James P. Elia on March 14, 1995. A copy of such disclosures is attached hereto as Exhibit D. I understand that the same disclosures are contained in above patent application Serial No. 09/064,000.

4. I note that the disclosures mentioned in above Paragraph 3 relate to a method for forming a bud and then for forming soft tissue. Such methods involve placing a growth factor at a desired site of a body with use of techniques including resorbable and non-resorbable carriers, gels, time-

release capsules, and granules. In addition, the growth factor may be placed in the body orally, systemically, by injection, through the respiratory tract, by making an incision in the body and then inserting the growth factor. I note further that the growth factor and/or carrier may be activated by tissue pH, enzymes, ultrasound, electricity, heat, or in vivo chemicals.

5. It is well known and established in the medical arts that buds are a primordium or, in other words, a rudiment or commencement of an organ. The process of organ formation includes the differential development of cells to form an organ primordium with the resulting formation of soft tissue. Such process of development is called organogenesis. It is also well known and established in the medical arts that the term "soft tissue" includes blood vessels.

In making the above statement in this Paragraph, I am aware of the definitions attached hereto as Exhibit B. Terms included in the above-mentioned definitions are: bud, primordium; organogenesis, and organ. I am also aware of and have considered the definition of "growth factor" as contained in Column 14 of the aforesaid '235 patent.

6. The materials included in attached Exhibit C evidence that the placement of growth factors in the body of a human results in the formation of a bud with subsequent growth into soft tissue. These materials report work performed by reputable, skilled scientists and reputable organizations in the medical arts. Consequently, I believe that these reports would be recognized as clearly valid by one of ordinary skill in the medical arts because they report the results of scientific tests conducted by competent, disinterested third parties with use of proper scientific controls.

7. Based upon the materials included in above Paragraphs 4, 5, and 6, it is my opinion that the process of placing a growth factor at a desired site of a human body will produce a bud that will predictably subsequently grow into soft tissue, as described in the '235 patent, using the techniques identified in above Paragraph 4. My further opinion is that when the techniques and angiogenic growth factors described and disclosed in the Elia patent application are used to place such growth factors in a human host, such placement would result in the formation of soft tissue, e.g., blood vessels. My opinion is in accord with the results obtained by the Isner patent (Exhibit C-6) which employed the same angiogenic growth factors and carrier/technique described and disclosed in the Elia patent application.

8. Declarant states that the above opinion was reached independently.

Declarant understands that (1) any willful false statements and the like made herein are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon, and (2) that all statements made of Declarant's own knowledge are true and that all statements made on information and belief are believed to be true.

Further Declarant sayeth not.

Date: 2/11/01

C. Gene Wheeler

C:\MYDOCUMENTS\CLIENTS\ELIA\WHEELERDECLARATION.DOC

EXHIBIT A

CURRICULUM
VITAE

C. GENE WHEELER, M.D., F.A.C.S.
6342 E. Hillcrest Blvd.
Scottsdale, Arizona 85251
Home Phone: (480) 941-8345

CURRICULUM VITAE

Clarence Gene Wheeler

Born: May 23, 1930
U.S. Citizen

Education: Bachelor of Science, Wheaton College, Wheaton, Illinois - August 1951 with highest honors
Baylor University College of Medicine, May 1955, M.D. with highest honors
Surgical Internship: Massachusetts General Hospital Boston, Massachusetts 1955-1956
Surgical Residency: Massachusetts General Hospital, Boston, Massachusetts 1956-1962
Cardiovascular Fellowship: Methodist Hospital, Houston, Texas; Dr. DeBakey and Dr. Cooley, September 1960 - June 1961

Academic: Prior: Clinical Associate Professor of Surgery, Southwestern Medical School, Dallas, Texas
Clinical Associate Professor of Surgery, University of Arizona College of Medicine, Phoenix Campus

Boards: American Board of Surgery 1963

Hospital Affiliations: Attending Surgeon, Baylor University Medical Center, Dallas, Texas, 1962 to June 1990 (resigned)
Chief, Vascular Surgery, Carl T. Hayden V.A Medical Center, Phoenix, Arizona 1990 - 1999

Organizations: American College of Surgeons 1966
The Cooley Cardiovascular Society
The DeBakey Cardiovascular Society
The International Cardiovascular Society
Texas Surgical Society
Phoenix Vascular Group - Co-founder 1991

Other Interests: Fishing, Skiing, Tennis, Security and Real Estate Investment
Missionary Surgery (Mexico, Central America, India)

Community Interests: The Episcopal Foundation, Terrell Branch, Terrell, Texas
Gideon International, Central Phoenix Branch, Phoenix, Arizona
Former Member, Vestry Board of the Good Shepherd Episcopal Church, Terrell, Texas
Former Member, Vestry of St. Matthew's Cathedral, Dallas, Texas

Community Interests Continued:

Former Member, Deacon Board of the First Baptist Church, Dallas, Texas
Former Trustee of Dallas Theological Seminary, Dallas, Texas
Former member, Vestry Board, Christ Church of Ascension - Episcopal, Paradise Valley, Arizona

Teaching Assignments:

Director of Vascular II Surgical Service, Baylor University Medical Center, Dallas, Texas 1970-1985
Director of Vascular Training Fellowship, Baylor University Medical School, 1968-1985
Founder of Visiting Vascular Lectureship, Baylor University Medical Center, Dallas, Texas 1978
Faculty Member, Phoenix Integrated Surgical Residency Program, 1990
Co-Director/Founder - National PACT Training Program, 1995

Administrative Experience:

Senior Partner and Manager, Surgical Associates of Dallas, Dallas, Texas, 1970-1985 - Surgical Practice and Financial Management Affairs
Member of Texas Medical Foundation Regional Quality Assurance Committee, Dallas, Texas, 1988-1989
Member, Infection Control Committee
Member, Quality Assurance Committee
Member, ICU-CCU Committee
Member, Hospital By-Laws Committee
Member, Pharmacy and Therapeutics Committee
Member, Institutional Review Board, Carl T. Hayden VAMC

Investigations in Progress:

Chief Investigator: C.G. Wheeler, M.D.

1. Safe Carotid Endarterectomy - Ongoing Quality Care Series 1990
2. Below Knee PTFE Reconstruction with AV Fistula in Diabetic Patients - IMPRA, Inc., 1993
3. Operation Desert Foot - Amputation Prevention Program, and Statistical Analysis, 1991 to present
4. TOPAZ Trial (Thrombolysis in Acute Arterial Occlusion) - Abbott Laboratories, 1993
5. PURPOSE Trial, Abbott Laboratories, 1997

Presentations:

Presiding Officer, Phoenix Vascular Group Vascular Lectureship, March 1991
Phoenix Integrated Surgical Residency Faculty lectures:
Acute Arterial Occlusion, 1991
Safe Carotid Endarterectomy, 1993
Surgical Considerations in Diabetic Ischemia, 1994
Texas Surgical Society:
Early Experiences in Distal Bypass Surgery for Patients with

EXHIBIT
B

DEFINITIONS

STEDMAN'S MEDICAL DICTIONARY

ILLUSTRATED

A vocabulary of medicine and its allied sciences, with pronunciations and derivations

TWENTY-SECOND EDITION

Completely revised by a staff of 33 editors, covering 44 specialties and subspecialties

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anxiety accompanying psychosomatic disorders; should not be used for nausea of pregnancy.

bud. A structure that resembles the b. of a plant.
bronchial b., one of the outgrowths from the primordial bronchus responsible for the continued ramification of the embryonic bronchial tree.
end b., tail b.
farcy b., one of a number of nodules formed along the course of the subcutaneous lymphatics in cases of glanders.
gustatory b., *caliculus* gustatorius.
liver b., the primordial cellular outgrowth from foregut entoderm of the embryo that gives rise to the parenchyma of the liver.
lung b., in the embryo, one of the two lateral outgrowths from the respiratory primordium that ultimately forms the epithelial portions of the lung.
metanephric b., ureteric b.; the primordial cellular outgrowth from the mesonephric duct that gives rise to the epithelial lining of the ureter, pelvis and calyces of the kidney, and the straight collecting tubules.
syncytial b., syncytial *knot*.
tail b., end b.; the rapidly proliferating mass of cells at the caudal extremity of the embryo.
taste b., *caliculus* gustatorius.
tooth b., the primordial structures from which a tooth is formed; the enamel organ, dental papilla, and the dental sac enclosing them.
ureteric b., metanephric b.
vascular b., an endothelial sprout arising from a blood vessel.

Budd, George, London physician, 1808–1882. See B.'s *cirrhosis, jaundice, syndrome*.

Budde (bood'deh), E., Danish sanitary engineer, *1871. See B. *process*.

buddeize (bood'de-ize) To treat by the Budde process.

budding. Gemmation.

Budge (bood'ga), Julius L., German physiologist, 1811–1888. See B.'s *center*.

Budin (bü-dăn'), Pierre C., French gynecologist, 1846–1907. See B.'s obstetrical *joint*, B.'s *pelvimeter*.

Buerger, Leo, New York physician, 1879–1943. See B.'s *disease*, Winiwarter-B. *disease*, B.'s *stain*.

bufa-, bufo-. Combining forms that denote origin from toads. They are used in the systematic and trivial names of a great number of toxic substances (genins) isolated from plants and animals containing the bufanolide structure (see bufanolide). Prefixes denoting species origin are often attached, *e.g.*, marinobufagin, marinobufotoxin.

bufagenins. Bufagins.

bufagins. Bufagenins; a group of steroids (bufanolides) in the venom of a family of toads, the Bufonidae, having a digitalis-like action upon the heart (*e.g.*, bufotalin); *cf.* bufotoxins. For structure of bufanolides, see steroids.

bufalin. A specific type of bufanolide, containing 3β,14-dihydroxy-5β,14β-bufa-20,22-dienolide. For structure of bufanolide, see steroids.

bufanolide. Fundamental steroid lactone of several squill-toad (Bufonidae) venoms or toxins; also found in the form of glycosides in plants (*cf.* digitalis). The steroid is essentially that of 5β-androstane, with a 14β-H. The lactone at C-17 is structurally related to —$CH(CH_3)CH_2CH_2CH_3$ radical attached to C-17 in the cholanes, and is in the same configuration as that of cholesterol (*i.e.*, 20*R*); in some species, b. is formed from cholesterol. Various b. derivatives having unsaturation in the lactone ring (20,22) or elsewhere (4) are known as bufenolides (one double bond), bufadienolides (*e.g.*, bufalin, telecinobufagin, marinobufagin, bufogenin B, bufotalin, bufotoxin), bufatrienolides (*e.g.*, scillarenin), etc. They have varying numbers of hydroxyl groups at positions 3, 5, 14, and 16, and these may be further substituted (*e.g.*, bufatalin, bufotoxin, gitoxigenin). For structure, see steroids.

buffer. 1. A mixture of an acid and its conjugate base (salt), such as H_2CO_3/HCO_3 : $H_2PO_4^-/H_2PO_4^=$, which when present in a solution reduces any changes in pH that would otherwise occur in the solution when acid or alkali is added to it. Thus the pH of the blood and body fluids is maintained virtually constant (pH 7.45) although acid metabolites are continually being formed in the tissues and $CO_2(H_2CO_3)$ is lost in the lungs. See also conjugate acid-base pair, under conjugate. 2. To add a b. to a solution and thus give it the property of resisting a change in pH when it receives a limited amount of acid or alkali.
b. capacity, the amount of hydrogen ion (or hydroxyl ion) required to bring about a specific pH change in a specified volume of a b. (see b. value).
b. pair, an acid and its conjugate base (anion).
secondary b., see Hamburger's *law*.
b. value, the power of a substance in solution to absorb acid or alkali without change in pH; this is highest at a pH equal to the pK of the acid of the b. pair (see b. capacity).
b. value of the blood, the ability of the blood to compensate for acid-alkali fluctuations without disturbance of the pH.

Buffon (boo'-fon), Compt de (Georges Louis Leclerc), French naturalist, 1707–1788. Published *Histoire Naturelle*. Some of his views on evolution and the origin of species anticipated Darwin by more than a hundred years.

buffy coat. Crusta inflammatoria; crusta phlogistica; the upper, lighter portion of the blood clot (coagulated plasma and white blood cells), occurring when coagulation is delayed so that the red blood cells have had time to settle a little; the portion of centrifuged, anticoagulated blood which contains leukocytes and platelets.

bufo-. See bufa-.

bufogenin B. A steroid toxin from Chinese toads; a 3β,14,16-trihydroxy-bufa-20,22-dienolide; *cf.* bufalin.

Bufonidae [L. *bufo*, toad]. A family of toads whose dermal glands secrete several kinds of pharmacologically active substances having a cardiac action similar to that of digitalis.

bufotalin. The steroid of a bufotoxin (bufogenin). It is bufogenin B acetylated at the C-16 OH.

bufoten'ine. Mappine; 3-(2-dimethylaminoethyl)indol-5-ol; *N,N*-dimethylserotin; a psychotomimetic agent isolated from the venom of certain toads. It raises the blood pressure by a vasoconstrictor action and produces psychic effects including hallucinations. It is also present in several plants and is one of the active principles of cohoba.

bufotox'in. Vulgarobufotoxin; a toxic substance in venom of *Bufa vulgaris*, the common European toad; bufotalin esterified with suberylarginine at C-14 OH group.

bufotox'ins. A group of steroid lactones (conjugates of bufogenins and suberylarginine at C-14) of digitalis present in the venoms of the Bufonidae. Their effects are similar to but weaker than those of the bufagins.

bug'gery [O.F. *bougre*, heretic]. Bestiality; sodomy.

Buhl (bool), Ludwig von, German pathologist, 1816–1880. See B.'s *disease*.

Buist, Robert C., Scottish obstetrician, 1860–1939. See B.'s *method*.

bulb [L. *bulbus*, a bulbous root]. 1. Any globular or fusiform structure. 2. *Medulla* oblongata. 3. A short, vertical underground stem of plants such as scilla and allium.
aortic b., *bulbus* aortae.
arterial b., *bulbus* aortae.
carotid b., *sinus* caroticus.
b. of corpus spongiosum, *bulbus* penis.
dental b., the papilla, derived from mesoderm, that forms the part of the primordium of a tooth which is situated within the cup-shaped enamel organ.
duode'nal b., duodenal *cap*.
end b., one of the oval or rounded bodies in which the sensory nerve fibers terminate in mucous membrane.
b. of eye, *bulbus* oculi.
hair b., *bulbus* pili.
ju'gular b., *bulbus* venae jugularis.
Krause's end b., *corpusculum* bulboideum.
b. of lateral ventricle, a rounded elevation in the dorsal part of the medial wall of the posterior horn of the lateral ventricle produced by the forceps major.
olfac'tory b., *bulbus* olfactorius.
b. of penis, *bulbus* penis.
rachid'ian b., *medulla* oblongata

Rouget's b., a venous pl
taste b., *caliculus* gustatc
b. of ure'thra, *bulbus* pe
b. of vestibule, *bulbus* v

bulbar. 1. Relating to a bull medulla oblongata.

bulbi'tis. Inflammation of

bulbocap'nine [G. *bolbos*, An alkaloid from *Corydalis* *Fumariaceae*). Produces a s recommended in the treatmer disease, paralysis agitans, an

bul'bocaverno'sus. See u

bulboid [G. *bolboeidēs*, fr resemblance]. Bulb-shaped.

bulbonu'clear. Relating to

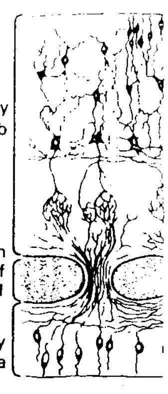
bulbopon'tine. Denoting t pons and the region of the m

bulbosac'ral. Relating to segments of the spinal cord.

bulbospin'al. Relating to th particularly to nerve fibers in

bulbourethral (bul'bo-u-re'

bulbus, gen. and pl. **bull**
b. aor'tae [NA], aortic bulb dilation where the truncus art
b. cordis, b. aortae
b. cornus posterior'is [N lateral ventricle of the brain; of the posterior horn produce of the corpus callosum as the lobe.
b. oc'uli [NA], bulb of the e proper without the appendage
b. olfacto'rius [NA], olf anterior extremity of the olfac plate of the ethmoid and rece



Bulbus O

Diagram of olfactory muc y Cajal), showing neuronal re Copenhaver, W. F.: *Bailey's* The Williams & Wilkins Co.,

b. penis [NA], bulb of corpu b. urethrae; the expanded poster penis lying in the interval betw
b. pili [NA], hair bulb; the lo that fits like a cap over the pap follicle.
b. ure'thrae, b. penis.
b. venae jugula'ris [NA], b dilated parts of the internal jugu at the beginning of the internal j

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Thesaurus

Go To

bud[1,noun]
bud[2,verb]
bud scale

Main Entry: [1]**bud**
Pronunciation: 'b&d
Function: *noun*
Etymology: Middle English *budde*
Date: 14th century
1 : a small lateral or terminal protuberance on the stem of a plant that may develop into a flower, leaf, or shoot
2 : something not yet mature or at full development: as **a :** an incompletely opened flower **b :** CHILD, YOUTH **c :** an outgrowth of an organism that differentiates into a new individual : GEMMA; *also* : PRIMORDIUM
- in the bud : in an early stage of development in the bud>

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Thesaurus Symbol Key

* generally or often considered vulgar
|| usage restricted; consult a dictionary for more information

For further explanation of these symbols see the Thesaurus Symbol Guide.

Dictionary Pronunciation Key

Merriam-Webster

WWWebster Dictionary

Thesaurus

Main Entry: **pri·mor·di·um**
Pronunciation: -dE-&m
Function: *noun*
Inflected Form(s): *plural* **pri·mor·dia** /-dE-&/
Etymology: New Latin, from Latin
Date: 1671
: the rudiment or commencement of a part or organ

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Thesaurus Symbol Key

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Dictionary Pronunciation Key

- \&\ as **a** and **u** in **abut**
- \$^{\&}$\ as **e** in **kitten**
- \&r\ as **ur** and **er** in **further**
- \a\ as **a** in **ash**
- \A\ as **a** in **ace**
- \ä\ as **o** in **mop**
- \au\ as **ou** in **out**
- \ch\ as **ch** in **chin**
- \e\ as **e** in **bet**
- \E\ as **ea** in **easy**
- \g\ as **g** in **go**
- \i\ as **i** in **hit**
- \I\ as **i** in **ice**
- \j\ as **j** in **job**
- \[ng]\ as **ng** in **sing**
- \O\ as **o** in **go**
- \o\ as **aw** in **law**
- \oi\ as **oy** in **boy**
- \th\ as **th** in **thin**
- \th\ as **th** in **the**
- \ü\ as **oo** in **loot**
- \u\ as **oo** in **foot**
- \y\ as **y** in **yet**
- \zh\ as **si** in **vision**

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organogenesis

organogenesis,

in embryology, the series of organized integrated processes that transforms an amorphous mass of cells into a complete organ in the developing embryo. The cells of an organ-forming region undergo differential development and movement to form an organ primordium, or anlage. Organogenesis continues until the definitive characteristics of the organ are achieved. Concurrent with this process is histogenesis; the result of both processes is a structurally and functionally complete organ. The accomplishment of organogenesis ends the period during which the developing organism is called an embryo and begins the period in which the organism is called a fetus. See also histogenesis.

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Thesaurus
organ
barrel organ
Go To electric organ

Main Entry: **or·gan**
Pronunciation: 'or-g&n
Function: *noun*
Etymology: Middle English, partly from Old English *organa,* from Latin *organum,* from Greek *organon,* literally, tool, instrument; partly from Old French *organe,* from Latin *organum;* akin to Greek *ergon* work -- more at WORK
Date: before 12th century
1 a *archaic* **:** any of various musical instruments; *especially* **:** WIND INSTRUMENT **b** (1) **:** a wind instrument consisting of sets of pipes made to sound by compressed air and controlled by keyboards and producing a variety of musical effects -- called also *pipe organ* (2) **:** REED ORGAN (3) **:** an instrument in which the sound and resources of the pipe organ are approximated by means of electronic devices (4) **:** any of various similar cruder instruments
2 a : a differentiated structure (as a heart, kidney, leaf, or stem) consisting of cells and tissues and performing some specific function in an organism **b :** bodily parts performing a function or cooperating in an activity organ*s*>
3 : a subordinate group or organization that performs specialized functions organ*s* of government>
4 : PERIODICAL

Dictionary Look Up: Search

Type in your word or phrase and click Search. Click on HELP for search tips.

Thesaurus Symbol Key

* generally or often considered vulgar
|| usage restricted; consult a dictionary for more information

EXHIBIT C

EXHIBIT C
SUMMARY OF MATERIALS

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-1	Science Daily (American Heart Association), 1998, "Study is first ever to document protein therapy induces creation of new blood vessels to the human heart" SYNOPSIS: For the first time ever, growth factor inserted into the body grows a new vascular system.	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)
C-2	Circulation, 1998, "Induction of neoangiogenesis in ischaemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease" SYNOPSIS: A new therapeutic concept and followup tests confirm a true de novo vascular system was formed . Vascular buds consisting of endothelial sprouts (capillaries) were created. The capillaries grew further and differentiated into two-layered metarterioles. The process of organogenesis continued with the metarterioles differentiating into three-layered arterioles (arteries).	Blood vessels to heart	Injection	Human recombinant basic fiberblast growth factor (genetically manipulated and produced)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-3	Circulation, 1998, Editorial, "Angiogenic therapy of the human heart" SYNOPSIS: Basic research in a different field (cancer) purified angiogenic growth factors in the 1980's. A novel clinical application of these growth factors introduces a new modality-the regulation of blood vessel growth.	Editorial	Editorial	Editorial
C-4	NIH Press Release: 1999, "Growing New blood vessels with a timed-release capsule of growth factor is a promising treatment for heart bypass patients, finds NHLBI Study" SYNOPSIS: Researchers at Harvard Mecial School inserted timed-release capsules of basic fibroblast growth factor into [human] heart muscle to grow new blood vessels.	Blood vessels to heart	Insertion of timed-release capsule	Basic fibroblast growth factor
C-5	The Lancet, 1996, "Clinical Evidence of angiogenesis after arterial gene transfer of phVEGF in Patient with Ischaemic limb" SYNOPSIS: Growth factor plus living material (plasmid) inserted into the body with a gel carrier to grow new blood vessels in the leg of a patient.	Blood vessels to leg	Balloon Catheter/ hydrogel	Vascular endothelial growth factor plus living material (plasmid)

EXH. NO.	MATERIAL AND DATE	SOFT TISSUE	TECHNIQUE	GROWTH FACTOR
C-6	U.S. Patent No. 5,652,225 (1997) Parent application filed 10/04/94 SYNOPSIS: The formation of new blood vessels in a human host by inserting a growth factor with a carrier into the body.	Formation of new blood vessels	Balloon catheter/ hydrogel	Angiogenic growth factors
C-7	Harvard University Gazette, 1998, "New Arteries Grown in Diseased Hearts" SYNOPSIS: Harvard Medical School researchers inject basic fibroblast growth factor through a carrier (tube) to grow new arteries in a human heart.	Formation of new arteries in hearts	Injection via tube (catheter); and implanted timed-release capsules	Basic fibroblast growth factor

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Source: ***American Heart Association (http://www.americanheart.org/)***

Date: Posted 3/2/1998

Study Is First Ever To Document Protein Therapy Induces Creation Of New Blood Vessels To The Human Heart

DALLAS, Feb. 24 -- For the first time, scientists have published research evidence that recombinant protein therapy can create new blood vessels to increase blood supply to the human heart. The report from German scientists appears in today's Circulation: Journal of the American Heart Association.

FGF-I, a human growth factor obtained through genetic engineering, was used in 20 patients with some form of ischemic or coronary heart disease, which results from blockages in the vessels leading to and from the heart. By injecting the growth factor near the blocked vessels, the scientists were able to induce neoangiogenesis -- the process by which the body can grow its own new capillary network to bypass occluded vessels.

"This capillary network is a true de novo vascular system," says Thomas-Joseph Stegmann, M.D., head of the department of thoracic and cardiovascular surgery at the Fulda Medical Center, Fulda, Germany. "We were able to use the recognized physiological effects of FGF-I to induce neoangiogenesis in the human ischemic heart."

As early as four days after application of FGF-I, the vascular structure around the diseased vessels was completely altered in all 20 of the patients. Like the spokes of a bicycle wheel, the new capillary vessels radiated outward from the point of injection, resulting in a twofold to threefold increase in blood flow to the heart, says the study's lead author.

Researchers found, on average, the ejection fraction of the 20 patients improved from 50.3 percent to 63.8 percent in the three years following the procedure. Ejection fraction measures how much blood leaves the

EXHIBIT C-1

heart with each beat and indicates how well the left ventricle -- the heart's main pumping chamber -- is functioning.

In follow-up angiographic imaging of the patients, it was clear that the growth factor injection had stimulated the creation of a new vascular system, says Stegmann. Three months after the procedure, he and his colleagues examined angiograms -- X-ray images of the heart -- of both the treated and control (untreated) patients and found that no blockages had formed in the new vessels.

All of the patients who received the FGF-I three years ago are still alive. The scientists report that no negative side effects have been seen in the patients who received the FGF-I.

Elizabeth Nabel, M.D., an American Heart Association board member, has done extensive research in gene and recombinant protein therapy over the past 12 years. She says this new research is encouraging for cardiovascular surgeons.

"It's a very important therapy for patients who have blocked arteries that are not amenable to bypass," says Nabel, professor of internal medicine and physiology and chief, division of cardiology at the University of Michigan. "This is not to say that bypass should be abandoned, but this research shows angiogenesis is a powerful therapy to be used with bypass surgery."

The procedure is still experimental, but scientists say the use of FGF-I may particularly benefit patients whose blocked vessels cannot be treated by cardiac bypass operations.

"At the moment, this procedure could not replace conventional bypass surgery," says Stegmann. "The question remains to be answered whether FGF-I or other growth factors are able to treat occlusions of greater coronary vessels, but currently, this is not possible."

Scientists have used gene therapy to grow vessels in other parts of the body -- such as in the legs in order to improve the health of patients who have blockages in lower leg blood vessels -- but this is the first published account of the use of recombinant protein therapy to induce angiogenesis in human hearts.

FGF-I was obtained from strains of Escherichia coli by genetic engineering, then isolated and highly purified the recombinant FGF-I protein. After several series of animal experiments demonstrated the potency of FGF-I, it was used in humans for the first time.

When scientists create recombinant protein, they take the DNA of a growth factor (in this case FGF-I) and manipulate it into RNA (ribonucleic acid) by growing it in bacteria cultures in the laboratory. RNA is then manufactured into protein, which is isolated and purified

before it is injected into the hearts of patients.

Twenty patients -- 14 men and 6 women who were at least 50 years old -- who had no prior history of heart attack or cardiac surgery had an operation to clear blockages in more than one vessel. All of them had stenosis -- narrowed blood flow due to atherosclerosis -- in their internal mammary artery/left anterior descending coronary artery. During the operative procedure, the growth factor protein -- in a dosage of 0.01 milligrams per kilogram of body weight -- was directly injected into the heart muscle near the blockage.

Prior to using the treatment in humans, the scientists performed several series of animal experiments, most specifically in ischemic rat hearts. Having found that the FGF-I injection worked in those animal models, the researchers theorized that it would also work in humans.

Study co-authors are P. Pecher, M.D.; B.U. von Specht, M.D. and B. Schumacher, M.D.

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Induction of Neoangiogenesis in Ischemic Myocardium by Human Growth Factors

First Clinical Results of a New Treatment of Coronary Heart Disease

B. Schumacher, MD; P. Pecher, MD; B.U. von Specht, MD; Th. Stegmann, MD

Background—The present article is a report of our animal experiments and also of the first clinical results of a new treatment for coronary heart disease using the human growth factor FGF-I (basic fibroblast growth factor) to induce neoangiogenesis in the ischemic myocardium.

Methods and Results—FGF-I was obtained from strains of *Escherichia coli* by genetic engineering, then isolated and highly purified. Several series of animal experiments demonstrated the apathogenic action and neoangiogenic potency of this factor. After successful conclusion of the animal experiments, it was used clinically for the first time. FGF-I (0.01 mg/kg body weight) was injected close to the vessels after the completion of internal mammary artery (IMA)/left anterior descending coronary artery (LAD) anastomosis in 20 patients with three-vessel coronary disease. All the patients had additional peripheral stenoses of the LAD or one of its diagonal branches. Twelve weeks later, the IMA bypasses were selectively imaged by intra-arterial digital subtraction angiography and quantitatively evaluated. In all the animal experiments, the development of new vessels in the ischemic myocardium could be demonstrated angiographically. The formation of capillaries could also be demonstrated in humans and was found in all cases around the site of injection. A capillary network sprouting from the proximal part of the coronary artery could be shown to have bypassed the stenoses and rejoined the distal parts of the vessel.

Conclusions—We believe that the use of FGF-I for myocardial revascularization is in principle a new concept and that it may be particularly suitable for patients with additional peripheral stenoses that cannot be revascularized surgically. **(*Circulation*. 1998;97:645–650.)**

Key Words: growth substances ■ angiogenesis ■ coronary disease

For the cardiac surgeon who is attempting to treat CHD, the use of sections of autologous blood vessels as bypass material is subject to severe limitations. Autologous arterial conduits are in short supply, and segments of the saphenous vein do not remain patent for very long.[1,2] Furthermore, "complete" revascularization is limited if diffuse coronary arteriosclerosis is present and extensive, especially if there are additional peripheral stenoses.

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In the search for alternative and/or additional treatment for improving the long-term prognosis, especially in diffuse CHD, attention has recently been directed toward natural angiogenesis.[3-9] Growth factors, especially FGF-I, have recently become of major importance because they can induce angiogenesis.[8,10-12]

Gimenez-Gallego et al[13] succeeded in elucidating the biochemical structure of FGF-I in 1985. Jaye et al[14] isolated human FGF-I from brain tissue in 1986. In 1991, Forough and coworkers[15] successfully used the technique of gene transfer to introduce the information for expressing human FGF-I into apathogenic *Escherichia coli*.

Our aim was to evaluate the information currently available on the biological effect of angiogenetic growth factors in animals and, if appropriate, to use human growth factor for the treatment of CHD. This involved (1) the production of human growth factor by genetic engineering, followed by its isolation, characterization, and purification; (2) using animal experiments to establish its angiogenetic potency and to exclude any possible pathogenic effect; and (3) using FGF-I clinically as an adjunct to coronary surgery and to demonstrate neoangiogenesis in the ischemic human myocardium.

Methods

Production and Purification of FGF-I

The production and purification of human FGF-I is a biochemically elaborate technique. The individual experimental steps have been reported elsewhere.[4,7]

Genetic engineering was used to produce human FGF-I from apathogenic strains of *E coli*, a plasmid containing the genetic information being introduced into the microorganisms.[15] These were kindly provided by Prof T. Maciag (Laboratory of Molecular Biology, American Red Cross, Rockville, Md). After production, FGF-I was eluted by heparin sepharose column chromatography, and several elution fractions were collected and purified by dialysis. Positive protein elution fractions were identified in the BIO-RAD assay[7] by SDS-PAGE,[16] and the biochemical isolation of FGF-I was confirmed by the Western blot method.[17] Further purification was obtained by HPLC.[18] The factors were lyophilized and stored at −32°C and diluted to 1 mL with NaCl solution containing 500 IU of heparin.

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Selected Abbreviations and Acronyms

CHD	= coronary heart disease
EDP	= electronic data processing
FGF	= basic fibroblast growth factor
HPLC	= high-pressure liquid chromatography
IMA	= internal mammary artery
LAD	= left anterior descending coronary artery

Chorioallantoic Membrane Assay

This established method, which provides a direct demonstration of the effect of growth factors on living tissue, was used to investigate the angiogenetic effect of FGF-I.[19,20] The growth of the allantoic systems can be directly observed by light microscopy. After incubation of 20 fertilized hen eggs for 13 days, the growth factor was applied to the membrane and covered with tissue culture coverslips. Four days later, the membrane was examined under the light microscope and directly compared with controls untreated with FGF-I or treated with heat-denatured FGF-I (70°C for 3 minutes).

Exclusion of the Pyrogenicity of FGF-I

Varying concentrations of FGF-I (0.01, 0.5, or 1.0 mg/kg body weight) were injected subcutaneously, intramuscularly, or intravenously into 27 New Zealand White rabbits, the solvent alone being used for an additional 13 controls. Thereafter, the rectal temperature was taken every half hour for 3 hours, hourly for the rest of the day, and every 8 hours for 12 days. A daily white cell count was also repeated for 12 days (see "Results"). In addition to this, the erythrocyte sedimentation rate and the C-reactive protein values were determined on the 3rd, 6th, 9th, and 12th days after the injection.

Confirmation of the Angiogenetic Potency of FGF-I in Animal Experiments

Supplementary to our earlier experiments,[4,7] the effect of FGF-I was also investigated in the ischemic hearts of inbred Lewis rats (a total of 275 animals, including 125 controls treated with heat-denatured FGF-I, 70°C for 3 minutes). The pericardium was opened via the abdominal wall and diaphragm, and two titanium clips were inserted at the apex of the left ventricle to induce myocardial ischemia. Growth factor (mean concentration of 10 μg) was then injected locally into the site. The coronary vessel system was imaged by aortic root angiography after 12 weeks and, finally, a specimen from the same myocardial region was evaluated histologically.

Clinical Use of FGF-I in Patients With CHD

This study was approved by the Medical Research Commission at the Phillips University of Marburg on August 10, 1993 (No. 47/93). This is the usual ethics commission for our hospital. Twenty patients without any history of infarction or cardiac surgery (14 men and 6 women; minimum age, 50 years) were subjected to an elective bypass operation for multivessel coronary heart disease. The growth factor was applied directly during the operation. As a control group, 20 patients who underwent the same procedure were given heat-denatured FGF-I (70°C for 3 minutes). The choice of treatment was completely random, the names being placed in sealed envelopes and selected in a blinded manner.

The details, nature, and aims of this procedure were explained beforehand to every patient who underwent the operation. In all cases, we received their fully informed consent. Both groups of patients were closely comparable with regard to clinical symptoms, accompanying disorders, cardiovascular risk factors, ventricular function, sex, and age. A comparable coronary morphology was found in both groups.

All patients had a further stenosis in the distal third of the LAD or at the origin of one of its branches in addition to a severe proximal stenosis. The mean ejection fraction of the left ventricle for all patients was 50%. The operative procedure for coronary revascularization with autologous grafts (an average per patient of 2 to 3 venous bypasses and 1 from the left IMA) was routinely performed. FGF-I (mean concentration, 0.01 mg/kg body weight) was injected into the myocardium, distal to the IMA/LAD anastomosis and close to the LAD, during the maintenance of the extracorporeal circulation and after completion of the distal anastomoses (Fig 1). In the control group, heat-denatured FGF-I was substituted for FGF-I. After 12 weeks, the IMA bypasses of all the patients were imaged selectively by transfemoral, intra-arterial, and digital subtraction angiography.

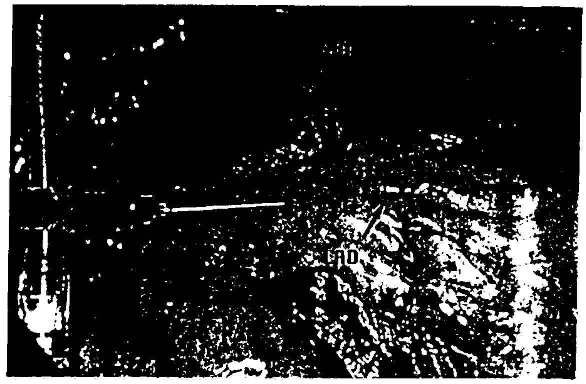


Figure 1. Intraoperative administration of growth factor.

Angiograms obtained in this way were evaluated by means of EDP-assisted digital gray-value analysis, a universally recognized and well-established technique for demonstrating capillary neoangiogenesis.[21-26] Sites of interest both with and without FGF-I (meaning heat-denatured FGF-I) were selected in the vessels filled with contrast medium and in regions of the myocardium distal to the IMA/LAD anastomosis. One hundred pixels were selected from each site of interest and analyzed digitally. Complete blackening of the x-ray films was rated with a gray value of 150, and areas without blackening of the film were allotted a zero value. During the first 5 postoperative days, separate laboratory checks in addition to the routine postoperative follow-up procedures were made twice daily, and the temperature checked three times a day.

Results

After separation, purification, and stabilization, we were able to isolate human FGF-I in all 40 bacterial cultures and demonstrate its high degree of purity. Fig 2 shows an HPLC profile of the growth factor after routine purification. The peak values at the beginning and end of the profile represent impurities that could be identified as *E coli* proteins. FGF-I could be further separated by fractionated collection, and the control HPLC (Fig 3) merely shows the peak value of this fraction on an otherwise even baseline.

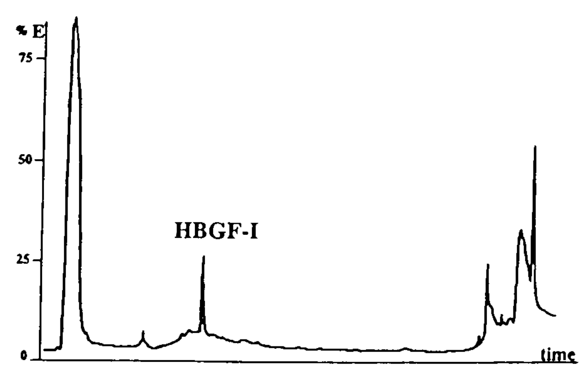


Figure 2. HPLC profile before high purification. HBGF-I indicates human FGF-I; %E, extinction.

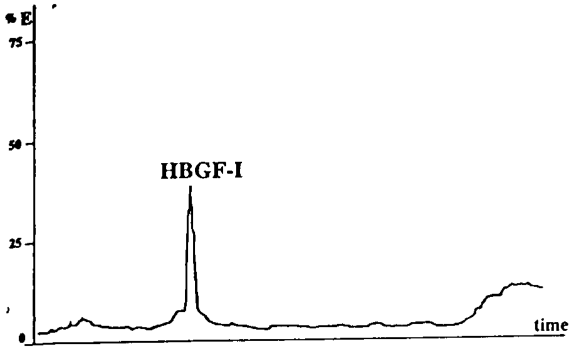


Figure 3. HPLC profile after high purification. HBGF-I indicates human FGF-I; %E, extinction.

In the chorioallantoic membrane assay, the angiogenetic potency of FGF-I could be demonstrated in vivo. As early as 4 days after application of the factor, the vascular structure of the membrane was completely altered. Emanating radially from the site of application, an unequivocal growth of new vessels from the original host vessels had grown out into the periphery (Fig 4A). These structures were completely absent from the control group, and a normally developed reticular vascular pattern could be discerned (Fig 4B).

Pyrogenic effects of the human growth factor produced in this way could be definitively ruled out in the animal model. There was no significant rise of body temperature when checked at short intervals and no trace of an inflammatory reaction in comparison with the control group (n=13) in any of the 27 test animals during the period of observation. This result was independent of the concentration and the route of administration (intravenous, subcutaneous, or intramuscular) of the factor.

Earlier investigations into the application of FGF-I to the nonischemic rat heart made it possible to demonstrate neoangiogenesis both histologically and angiographically after 9 weeks in 11 of 12 test animals after the implantation of a tissue bridge pretreated with growth factor between the heart and thoracic aorta. In the control group without FGF-I (n=6), no signs of induced neoangiogenesis could be found.[4,7]

Unequivocal proof of induced neoangiogenesis was also found in the ischemic rat heart. In the test animals, in which myocardial ischemia had previously been induced with titanium clips and growth factor had subsequently been injected into the myocardium, a manifest accumulation of contrast medium was shown by aortic angiography at the site of the FGF-I injection 12 weeks later (Fig 5A), whereas such an accumulation of contrast medium did not appear in any of the control animals (Fig 5B). Histological examination of the myocardium revealed a threefold increase in the capillary density per square millimeter around the site of the FGF-I injection.

When the growth factor FGF-I was used clinically for the first time on the human heart, neoangiogenesis together with the development of a normal vascular appearance could be demonstrated angiographically, exactly as in the earlier animal experiments.[4,7] Selective imaging of the IMA bypasses by intra-arterial digital subtraction angiography confirmed the following result in all 20 patients: at the site of injection and in the distal areas supplied by the LAD, a pronounced accumulation of contrast

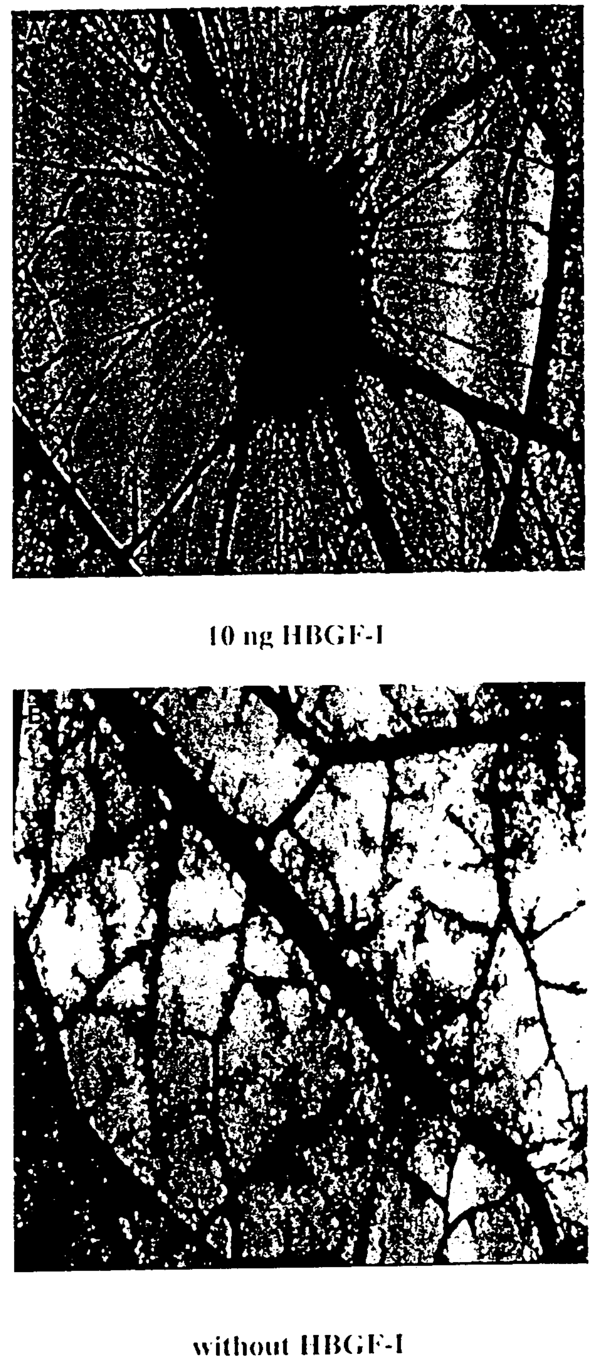


Figure 4. A, Chorioallantoic membrane assay with application of the growth factor. B, Chorioallantoic membrane assay of the control group. HBGF-I indicates human FGF-I.

medium extended peripherally around the artery for ≈3 to 4 cm, distal to the IMA/LAD anastomosis (Fig 6A). In the control angiograms of patients to whom only heat-denatured FGF-I had been given, the IMA/LAD anastomosis was also recognizable, but the accumulation of contrast medium described above was absent (Fig 6B). The angiograms of both the treated and control groups were recorded at a rate of four images per second, and these show

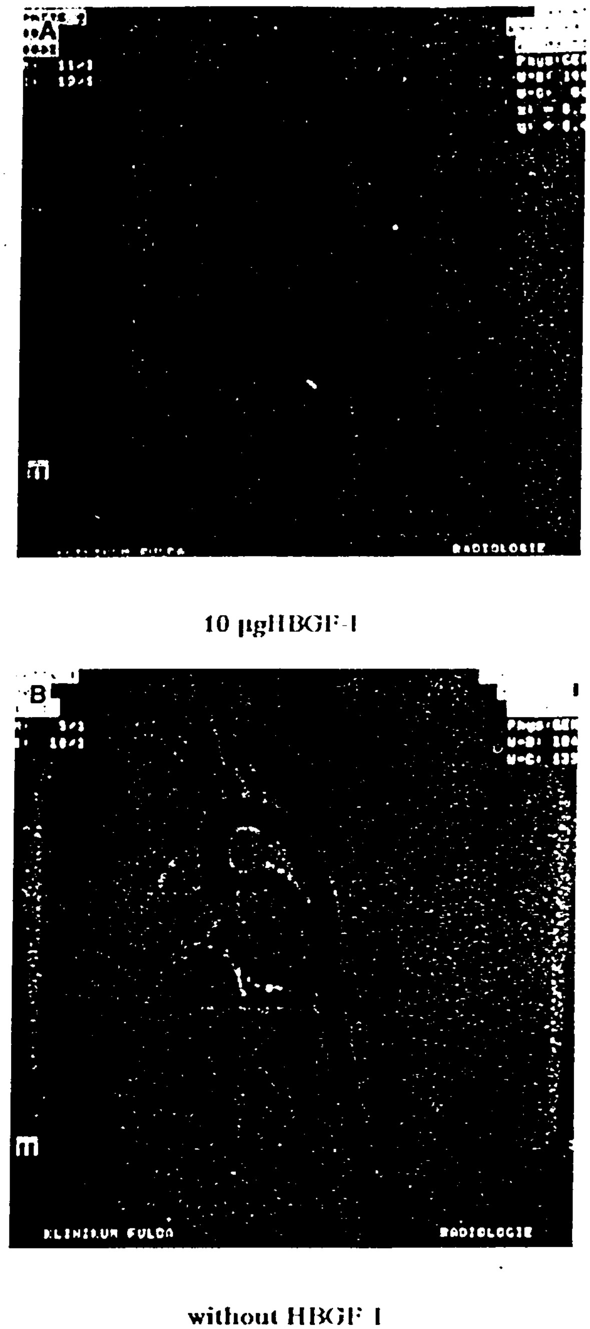


Figure 5. A, Administration of the growth factor in ischemic rat heart with a clearly discernible accumulation of contrast medium at the site of injection. B, No discernible accumulation of contrast medium in the control group. HBGF-I indicates human FGF-I.

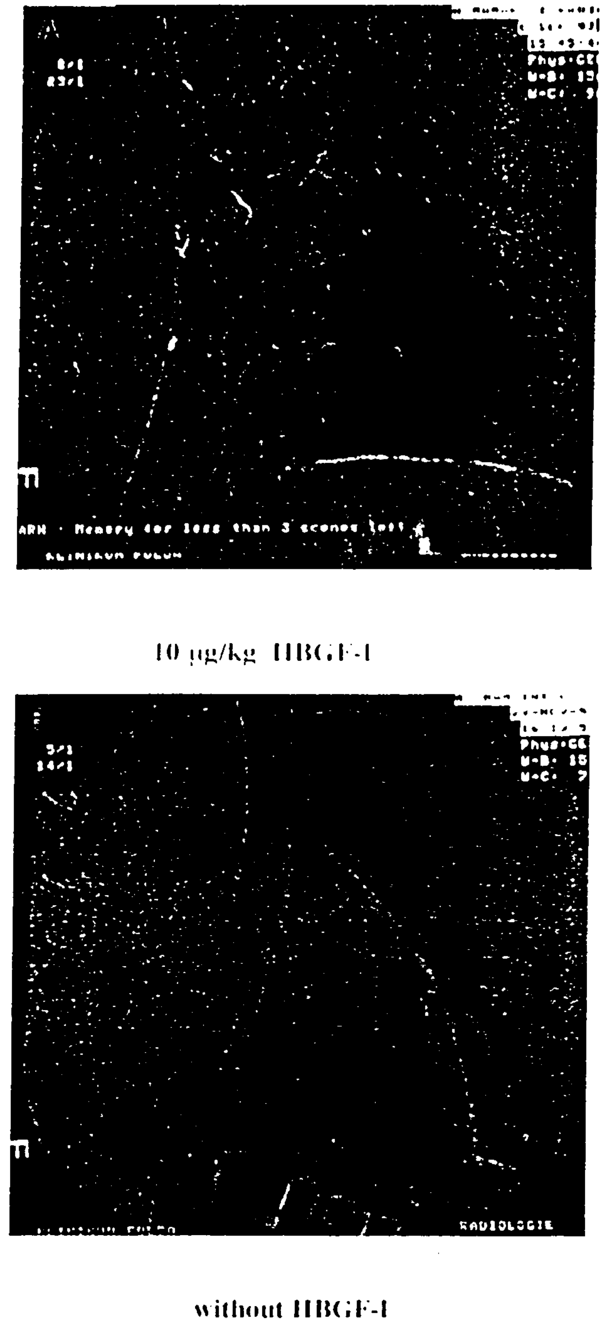
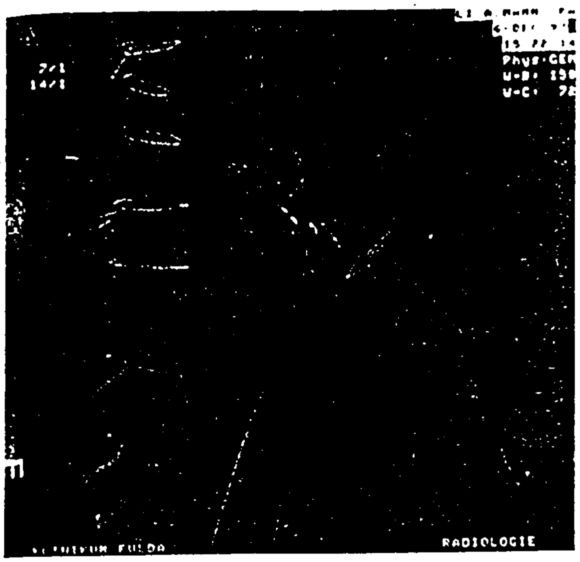


Figure 6. A, Angiography after injection of the growth factor into the human heart shows a pronounced accumulation of contrast medium compared with the control group. B, Angiography in the control group does not show any increased accumulation of contrast medium around the IMA/LAD anastomosis. HBGF-I indicates human FGF-I.

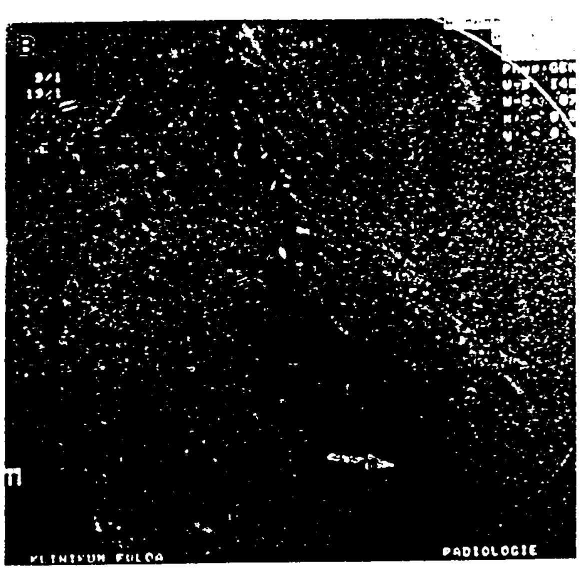
comparable distances between the beginning of the injection and visualization of the medium.

At the site of injection of the FGF-I, a capillary network could be seen sprouting out from the coronary artery into the myocardium. This enabled retrograde imaging of a stenosed diagonal branch to be performed (Fig 7A). Such "neocapillary vessels" can also provide a collateral circulation around additional distal stenoses of the LAD (Fig 7B) and bring about retrograde filling of a short segment of the artery distal to the stenosis. In none of the angiograms of the treated patients taken 12 weeks after the operation were any new stenoses of the LAD detectable.

The results of EDP-assisted digital gray value analysis for quantification of the neoangiogenesis (Fig 8) gave a mean gray value of 124 for the vessels. The control myocardium reached



10 μg/kg HBGF-I



10 μg/kg HBGF-I

Figure 7. A, Collateralization of stenoses (arrow): a diagonal branch occluded just distal to its origin is filled through the newly grown capillaries. B, Collateralization of stenoses (arrow) by newly grown capillaries: the peripherally stenosed LAD is filled through these vessels. HBGF-I indicates human FGF-I.

a gray value of only 20, and that of the myocardium injected with FGF-I gave a value of 59 (Fig 8).

Discussion

Normal capillaries have a cell population with a low turnover rate of months or years. On occasion, however, a high turnover rate of this cell population is possible even under physiological conditions, and this naturally leads to the rapid growth of new capillaries and other blood vessels. Such a physiological process occurs in the development of the placenta, in fetal growth, and in wound healing, as well in the formation of collaterals in response to tissue ischemia. "Angiogenetic growth factors," which are biochemically polypeptides, are essential for such processes as capillary growth or neoangiogenesis. These growth factors (for instance, the human heparin-binding FGF-I) bring about their effect by significantly increasing cell proliferation, differentiation, and migration via a high-affinity receptor system on the surfaces of the endothelial cells.[8,10–12]

During the last few years, several working groups have been able to establish indications for the effective use of growth factors to improve blood flow in the presence of tissue ischemia in animal experiments.[3,9,27] Yanagisawa-Miwa et al[9] succeeded in demonstrating a significant collateralization together with reduction in the size of the infarct after intracoronary administration of growth factor in rabbits. Baffour et al[3] also observed a significant formation of collaterals in ischemic extremities after growth factor administration in animals. Albes et al[27] produced a distinct improvement in the blood flow in ischemic tracheal segments implanted subcutaneously in rabbits by injecting growth factor–enriched fibrin glue locally.

After growth factor was injected into the ischemic rat heart,[4,7] we were able to observe induced neoangiogenesis and confirm it angiographically. We were also able to prove histologically that this neoangiogenesis brings about the development of new vascular structures with a three-layered vessel wall. Angiographic imaging confirmed that these are anatomically normal capillaries and other blood vessels.

The production of human FGF-I by our molecular biological method has proved to be a complex but readily reproducible procedure. From the bacterial cultures, we are able to isolate the factor as a pure substance in sufficient quantities. By in vitro assay and as a result of extensive animal experiments, we were able to exclude the possible pyrogenic effects of FGF-I.

In earlier animal experiments,[4] we were able to demonstrate the proliferative and mitogenic effects of the growth factor on human saphenous vein endothelial cells. Endothelial cell cultures with added growth factor induced a confluent monolayer after only 5 to 9 days, whereas the monolayer was not complete before 7 to 11 days in the control group. In addition to determining the total cell count with a cell counter, we also confirmed this result by analyzing the rate of DNA synthesis by measuring the incorporation of ^{3}H-thymidine into the endothelial cell nuclei using the

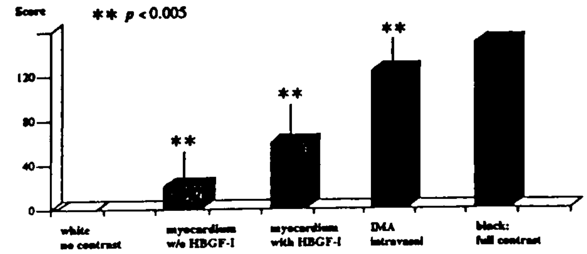


Figure 8. Quantitative gray value analysis of contrast medium accumulation in the angiography shows a twofold to threefold increase in the local blood flow at the site of injection. HBGF-I indicates human FGF-I.

method of Klagsbrun and Shing.[28] The cell proliferative potency of FGF-I could be further intensified by adding heparin, a glycosaminoglycan protecting the growth factor from inactivation by cellular enzymes and from heat and chemical denaturation.[29]

On the basis of these in vitro and in vivo experiments, we established for the first time the efficacy of FGF-I for the treatment of CHD, and were able to demonstrate that it can induce neoangiogenesis in situ in the ischemic human heart. This possibility has been widely discussed for many years but never before attempted.

A dense capillary network appeared around the site of injection of the factor in the myocardium of all our treated patients. This capillary network is a true de novo vascular system. Emerging from the proximal segment of the LAD, it sprouts out into the surrounding myocardium, bringing about a twofold to threefold increase in the local blood supply through these newly formed functional vessels. We were able to use the recognized physiological effects of FGF-I (as they occur in the repair mechanism of wound healing or in collateralization of ischemic tissue) to induce neoangiogenesis in the human ischemic heart.

We also consider that administration of FGF-I (produced in this way by genetic engineering), combined with operative myocardial revascularization, may well be an especially appropriate treatment for patients with additional peripheral stenoses that cannot be treated surgically.

In our opinion, neoangiogenesis induced by FGF-I opens up new possibilities for the treatment of ischemic myocardial disease. Furthermore, it could become a new therapeutic concept in the management of diffuse CHD after alternative methods of administration have also been developed. This method of inducing neoangiogenesis is also conceivable as a therapeutic option in other regions of the cardiovascular system in which arterial occlusion has led to ischemia.[30] However, before any such possibilities are realized, many more clinical investigations will have to be performed.

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EXHIBIT C-3

Editorial

Angiogenic Therapy of the Human Heart

Judah Folkman, MD

The field of angiogenesis research was initiated 27 years ago by a hypothesis that tumors are angiogenesis-dependent.[1] Shortly thereafter, in the early 1970s, it became possible to passage vascular endothelial cells in vitro for the first time.[2] Bioassays for angiogenesis were developed subsequently throughout that decade. The early 1980s saw the purification of the first angiogenic factors.[3–6] By the mid-1980s, angiogenesis inhibitors began to be discovered.[7–9] Translation of these laboratory findings to clinical application started in 1989, when interferon alfa was first used for the treatment of life-threatening hemangiomas in infants.[10–12]

See p 645

Clinical applications of angiogenesis research are being pursued along three general lines: (1) prognostic markers in cancer patients,[13,14] (2) antiangiogenic therapy (for review, see Reference 15), and (3) angiogenic therapy. The first angiogenic therapy of ischemic vascular disease was the administration of vascular endothelial growth factor (VEGF)/vascular permeability factor to patients with severe peripheral vascular disease in the lower limbs.[16]

In a landmark paper, Schumacher and colleagues now report the first angiogenic therapy of human coronary heart disease.[17] It is an important study, not only because the authors describe how they produced their own recombinant human fibroblast growth factor-1 (FGF-1, also called acidic fibroblast growth factor) and tested it in vitro and in vivo but also because they conducted a randomized controlled clinical trial. In 20 patients with three-vessel coronary artery disease who underwent two or three venous bypass grafts and one from the internal mammary artery, the angiogenic protein FGF-1 was injected into the myocardium close to the left anterior descending coronary artery and distal to its anastomosis with the internal mammary artery. FGF-1 was injected during extracorporeal surgery and again after completion of the anastomosis. Transfemoral, intra-arterial digital subtraction angiography 12 weeks later showed coronary artery neovascularization extending out from the area of FGF-1 injection. Stenoses distal to the anastomosis were bridged by neovascularization. This was similar to the neovascularization observed by the authors in rat hearts injected with FGF-1. Histological sections of rat myocardium showed a threefold increase in microvessel density. In 20 patients undergoing similar coronary artery bypass surgery in whom inactivated FGF-1 was injected, there was no evidence of myocardial neovascularization on the 12-week angiogram.

An advantage of this approach is that it induces local angiogenesis and appears to avoid high levels of circulating angiogenic activity that could possibly stimulate plaque angiogenesis and secondary plaque growth. Why does neovascularization persist for at least 12 weeks after only a single set of intramyocardial injections of the angiogenic protein? Perhaps persistent neovascularization was facilitated by upregulation of VEGF and its receptors in hypoxic tissue.[18] Furthermore, basic FGF and VEGF are synergistic mitogens for endothelial cells in vitro.[19,20] Also, FGF can increase expression of (or mobilize) VEGF.[21]

This report uses primarily anatomic studies to demonstrate increased myocardial neovascularization after angiogenic therapy. We look forward to the follow-up of these patients to learn whether they have significant functional improvement compared with the control group of patients who received inactive FGF. It may be difficult to discriminate the extent to which functional improvement is due to the angiogenic therapy per se, despite use of a control group, because of the concomitant internal mammary artery anastomosis and the relatively small number of patients in this study. Nevertheless, the angiographic documentation of myocardial revascularization suggests that functional improvement should follow.

Although major therapeutic advances in cardiology have been based on the general principles of control of blood pressure, regulation of cardiac rhythm, enhancement of myocardial contractile strength, increased diameter of narrowed coronary arteries, and lysis of intravascular thromboses, the report by Schumacher et al introduces a new modality, the regulation of blood vessel growth. If angiogenic therapy of the myocardium continues to live up to its potential as indicated by this report, we may witness novel refinements in future years as the molecular biology of endothelial cell and smooth cell growth is gradually uncovered. For example, the therapeutic induction of coronary arterial collaterals may someday be optimized by administration of appropriate mixtures of molecules that target different components of the vasculature, ie, the FGFs are mitogenic for vascular endothelial cells and smooth muscle, VEGF[22] is mitogenic primarily for endothelial cells, angiopoietin-1 mediates the recruitment of smooth muscle cells to the wall of new vessels,[23] and angiopoietin-2 appears to prevent or downregulate smooth muscle apposition to the walls of microvessels.[24] It is interesting that the methodology to discover these different vascular cell growth proteins emerged largely from investigations of mechanisms of tumor angiogenesis in studies funded primarily by the National Cancer Institute over many years. The report by Schumacher et al illustrates how unpredictable are the clinical applications that may arise from basic research in a different field.

The opinions expressed in this editorial are not necessarily those of the editors or of the American Heart Association.

From Children's Hospital, Harvard Medical School, Boston, Mass.
Correspondence to Judah Folkman, MD, Children's Hospital, Harvard Medical School, Hunnewell 103, 300 Longwood Ave, Boston, MA 02115. (*Circulation*. 1998;97:628-629.)

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KEY WORDS: Editorials ■ angiogenesis ■ growth substances

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EXHIBIT C-4

Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study

By The National Heart, Lung, and Blood Institute

Heart bypass patients treated with a timed-release capsule of a substance that promotes the growth of new blood vessels showed evidence of improved blood supply and heart function, according to a study supported by the National Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health.

"Growing" blood vessels, a strategy called angiogenesis, is a promising experimental treatment for blocked arteries in bypass surgery patients for whom surgery alone would not adequately restore blood flow to the heart.

Dr. Michael Simons and colleagues at Harvard Medical School inserted timed-release capsules of basic fibroblast growth factor (bfGF) into the heart muscle of patients scheduled for bypass surgery. Patients received either a 10 microgram (mcg) or 100 mcg dose

of the substance. Other patients received a harmless placebo capsule at the time of surgery. The relatively small study (24 patients total) was designed to test the safety and effectiveness of the procedure.

The study, published in the November 2, 1999 issue of *Circulation*, found that there were no serious adverse effects of the treatment. Both magnetic resonance imaging (MRI) and nuclear stress testing were used to evaluate changes in blood flow. Stress tests showed a worsening of blood flow in the placebo group, no change in the 10 mcg. group and significant improvement in patients receiving 100 mcg. MRI results showed clear improvement in blood flow in patients given 100 mcg. Patients in the highest dose group were free of angina (chest pain) but some patients in the placebo and low-dose group experienced chest pain.

Simons and colleagues note that a larger (Phase II) multi-center study of this approach is currently underway.

The National Heart, Lung, and Blood Institute of The National Institutes of Health. Press Release: **Growing New Blood Vessels with a Timed-Release Capsule of Growth Factor is a Promising Treatment for Heart Bypass Patients, Finds NHLBI Study**. November 1, 1999. (Online) http://www.nih.gov/news/pr/nov99/nhlbi-01.htm

United States Patent **5,652,225**
Isner **July 29, 1997**

Methods and products for nucleic acid delivery

Abstract

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, including antisense DNA or RNA. The nucleic acid may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one would select a DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

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Parent Case Text

This is a continuation of application Ser. No. 08/318,045 filed on Oct. 4, 1994 now abandoned.

Claims

1. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with a first DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial

growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a first DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said anglogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation.

2. The method of claim 1, wherein the angiogenic protein is vascular endothelial growth factor.

3. The method of claim 1, wherein the hydrogel polymer is selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides.

4. The method of claim 1, wherein the hydrogel polymer is a polyacrylic acid polymer.

5. The method of claim 1, wherein the hydrogel polymer is admixed with a second DNA encoding an angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor and having an operably linked secretory signal sequence or a second DNA encoding a modified angiogenic protein selected from the group consisting of acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor .alpha. and .beta., platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor .alpha., hepatocyte growth factor and insulin like growth factor having an operably linked secretory signal sequence, wherein said angiogenic protein induces new blood vessel formation when expressed in said target tissue in an amount effective to induce new blood vessel formation, and wherein said second DNA is not the same as said first DNA.

6. A method for inducing the formation of new blood vessels in a desired target tissue in a human host, comprising contacting an arterial cell in an artery or blood vessel via a balloon catheter coated with a hydrogel polymer admixed with DNA encoding vascular endothelial growth factor and which is expressed in an amount effective to induce new blood vessel formation.

Description

FIELD OF THE INVENTION

The present invention relates to delivery of nucleic acid to arterial cells and compositions therefor.

BACKGROUND OF THE INVENTION

Work from several laboratories (Nabel, et al., Science, 249:1285-1288 (1990); Lim, et al., Circulation, 83:2007-2011 (1991); Flugelman, et al., Circulation, 85:1110-1117 (1992); Leclerc, et al., J. Clin. Invest., 90:936-944 (1992); Chapman, et al., Circ. Res., 71: 27-33 (1992); Riessen, et al., Hum. Gene Ther., 4: 749-758 (1993); and Takeshita, et al., J. Clin. Invest., 93:652-661 (1994), has demonstrated

that recombinant marker genes could be transferred to the vasculature of live animals.

Gene delivery systems employed to date have been characterized by two principal components: a macrodelivery device designed to deliver the DNA/carrier mixture to the appropriate segment of the vessel, and microdelivery vehicles, such as liposomes, utilized to promote transmembrane entry of DNA into the cells of the arterial wall. Macrodelivery has typically been achieved using one of two catheters initially developed for local drug delivery: a double-balloon catheter, intended to localize a serum-free arterial segment into which the carrier/DNA mixture can be injected, or a porous-balloon catheter, designed to inject gene solutions into the arterial wall under pressure. Jorgensen et al., Lancet 1:1106-1108, (1989); Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-485 (1990); March et al., Cardio Intervention, 2:11-26 (1992)); WO93/00051 and WO93/00052.

Double balloon catheters are catheters which have balloons which, when inflated within an artery, leave a space between the balloons. The prior efforts have involved infusing DNA-containing material between the balloons, allowing the DNA material to sit for a period of time to allow transfer to the cells, and then deflating the balloons, allowing the remaining genetic material to flush down the artery. Perforated balloons are balloons which have small holes in them, typically formed by lasers. In use, fluid containing the genetic material is expelled through the holes in the balloons and into contact with the endothelial cells in the artery. These gene delivery systems however, have been compromised by issues relating to efficacy and/or safety.

Certain liabilities, however, inherent in the use of double-balloon and porous balloon catheters have been identified. For example, neither double-balloon nor porous balloon catheters can be used to perform the angioplasty itself. Thus, in those applications requiring both angioplasty and drug delivery, e.g., to inhibit restenosis, two procedures must be preformed. Additionally, the double balloon typically requires long incubation times of 20-30 min., while the high-velocity jets responsible for transmural drug delivery from the porous balloon catheter have been associated with arterial perforation and/or extensive inflammatory infiltration (Wolinsky, et al., J. Am. Coil. Cardiol., 15:475-481 (1990).

SUMMARY OF THE INVENTION

It has now been discovered that nucleic acids can be delivered to cells of an artery or blood vessel by contacting the cells with a hydrophilic polymer incorporating the nucleic acid, thus avoiding the use of a double-balloon or porous balloon catheter and the problems associated with such delivery systems. It has also been demonstrated that, unexpectedly, the percentage of transduced arterial cells is significantly higher using the present invention compared with use of a double-balloon catheter.

By "arterial cells" is meant the cells commonly found in mammalian arteries, including endothelial cells, smooth muscle cells, connective tissue cells and other cells commonly found in the arterial structure.

By "nucleic acid" is meant DNA and RNA, including antisense DNA or RNA.

It has further been discovered that a DNA encoding an angiogenic protein (a protein capable of inducing angiogenesis, i.e., the formation of new blood vessels), delivered by the method of the present invention is expressed by the arterial cell and induces angiogenesis in tissues perfused by the treated blood vessels. This allows for the treatment of diseases associated with vascular occlusion in a variety of target tissues, such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention provides a method for the delivery of a nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid. The nucleic acid may be any nucleic acid, DNA and RNA, including antisense DNA or RNA. The DNA may encode hormones, enzymes, receptors or drugs of interest. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, the genetic material of choice is DNA encoding an angiogenic protein. The nucleic acid may be carried by a microdelivery vehicle such as cationic liposomes and adenoviral vectors. DNA encoding different proteins may be used separately or simultaneously.

The hydrophilic polymer is selected to allow incorporation of the nucleic acid to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell. Preferably, the hydrophilic polymer is a hydrogel polymer. Other hydrophilic polymers will work, so long as they can retain the genetic material of the present invention, so that, on contact with arterial cells, transfer of genetic material occurs.

Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. The hydrogel polymer is preferably polyacrylic acid.

Without wishing to be bound by theory, one reason that the use of hydrogel, and particularly with hydrogel coated balloon catheters, is believed to provide improved results over, for example, prior treatments with double balloon catheters, is that the use of standard balloon catheters with hydrogel surfaces causes the hydrogel not only to contact the endothelial cells which line the interior of the arteries, but also displaces the endothelial cells sufficiently to permit contact between the hydrogel and the smooth muscle cells which underlie the endothelial cell layer. This permits expression of polypeptides in different arterial cell types, which enhances the kinds and amounts of therapeutic polypeptides which can be produced in accordance with this invention. For example, as indicated in the examples below, the present method successfully produces sufficient amounts of vascular endothelial growth factor (VEGF) to cause angiogenesis downstream from a DNA/arterial contact point, despite the fact that VEGF is not normally produced even by transformed endothelial cells, but is produced by smooth muscle cells of the type that surround the endothelial cells in the artery.

The arterial cell may be contacted with the hydrophilic polymer incorporating the DNA by means of an applicator such as a catheter which is coated with the DNA-bearing hydrophilic polymer. Preferably, the applicator can exert some pressure against the arterial cells, to improve contact between the nucleic acid-bearing hydrophilic polymer and the arterial cells. Thus a balloon catheter is preferred. Preferably, the hydrophilic polymer coats at least a portion of an inflatable balloon of the balloon catheter.

The present invention further includes compositions comprising hydrophilic polymers incorporating nucleic acid. Preferably the hydrophilic polymer is a hydrogel and the nucleic acid is DNA which encodes an angiogenic protein.

The present invention also provides kits for application of genetic material to the interior of an artery or similar bodily cavity, comprising a substrate, such as a catheter or a suitably shaped rod, and a source of genetic material comprising the DNA coding for the desired therapeutic polypeptide. Preferably, the present invention is directed to a catheter adapted for insertion into a blood vessel, having a balloon element adapted to be inserted into the vessel and expandable against the walls of the

vessel. At least a portion of the balloon element is defined by a coating of a hydrophilic polymer, and incorporated within the hydrophilic polymer coating, a nucleic acid to be delivered to the arterial cell. The hydrophilic polymer is preferably a hydrogel polymer, most preferably a hydrophilic polyacrylic acid polymer.

The present invention also provides a method for inducing angiogenesis in a desired target tissue, comprising delivering a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue.

Other aspects of the invention are discussed infra.

As used herein the term "angiogenic protein" means any protein, polypeptide, mutein or portion thereof that is capable of inducing the formation of new blood vessels. Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.alpha. and TFG-.beta.), platelet-derived endothelial growth factor (PD-ECGF), platelet-derived growth factor (PDGF), tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. Preferably, the angiogenic protein contains a secretory signal sequence allowing for secretion of the protein from the arterial cell. VEGF is a preferred protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1(a) and 1(b) show the rabbit ischemic hindlimb model. FIG. 1(a)is a representative angiogram recorded 10 days after surgery. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (arrow). Open arrow indicates the site of arterial gene transfer. In FIG. 1(b) the shaded segment of femoral artery has been excised.

FIGS. 2(a), 2(b) and 2(c) illustrate (a) RT-PCR analysis of transfected arteries, (b) Southern blot analysis of RT-PCR products and (c) nucleotide sequence of the RT-PCR product from transfected rabbit lilac artery. In FIGS. 2(a) and 2(b) the expression of the human VEGF mRNA was evident in the rabbit lilac artery (lane 4) and cultured rabbit vascular smooth muscle cells (lane 6, positive control) which were transfected with human VEGF gene. Arrows indicate position of VEGF band at 258 bp. Lane 1 depicts the results using a molecular weight marker, namely pGEM3zf(-) digested with Hae III; lane 2 is a negative control (no RNA); lane 3 is a second negative control (rabbit lilac artery transfected with .beta.-galactosidase expression plasmid); and lane 5 is a further negative control (PCR analysis of the VEGF-transfected lilac artery excluding the reverse transcriptase reaction). FIG. 2(c) shows the nucleotide sequence of the RT-PCR product from a transfected rabbit lilac artery. Direct sequencing of the 258 bp bands obtained by RT-PCR confirmed that this band represented the human VEGF sequence. The sequence designated in 2(c) corresponds to amino acids 69 to 75 of the VEGF peptide. Asterisks denote the nucleotides which are not conserved among different species of the VEGF gene (rat, mouse, bovine, guinea pig) demonstrating that the exogenous human gene was amplified by the RT-PCR procedure.

FIGS. 3A, 3B, 3C, 3D, 3E and 3F comprise internal lilac angiography of a control rabbit at (A) day 0 (pre-transfection), (B) day 10, and (C) day 30 post-transfection, and of a VEGF-transfected rabbit at (D) day 0, (E) day 10, and (F) day 30 post-transfection. In contrast to the control, angiographic examination of the VEGF-transfected animal discloses extensive collateral artery formation.

FIGS. 4(a), 4(b) and 4(c) are graphs illustrating the effect of VEGF-transfection on revascularization

in an ischemic limb model. FIG. 4(a) the angiographic score at day 0 (immediately prior to transfection), and days 10 and 30 post-transfection. FIG. 4(b) Calf Blood pressure ratio at day 0, and at days 10 and 30 post-transfection. FIG. 4(c) depicts capillary density at day 30 post-transfection. (*p<0.05,**p<0.01)

FIGS. 5(a) and 5(b) illustrate alkaline phosphatase staining of ischemic hindlimb muscle, counterstained with eosin. FIG. 5(a) depicts the muscle of an animal transfected with pGSVLacZ. FIG. 5(b) depicts the muscle of an animal transfected with phVEGF.sub.165. The dark staining indicates capillaries as shown by the arrows.

FIG. 6 illustrates a diagrammatical cross section of a balloon catheter having a hydrophilic surface bearing genetic material in accordance with the present invention, in place within an artery.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for the delivery of nucleic acid to an arterial cell comprising contacting the cell with a hydrophilic polymer incorporating the nucleic acid.

The nucleic acid may be any nucleic acid which when introduced to the arterial cells provides a therapeutic effect. The nucleic acid is selected based upon the desired therapeutic outcome. For example, in the treatment of ischemic diseases, one genetic material of choice would be a DNA encoding an angiogenic protein. DNA useful in the present invention include those that encode hormones, enzymes, receptors or drugs of interest. The DNA can include genes encoding polypeptides either absent, produced in diminished quantities, or produced in mutant form in individuals suffering from a genetic disease. Additionally it is of interest to use DNA encoding polypeptides for secretion from the target cell so as to provide for a systemic effect by the protein encoded by the DNA. Specific DNA's of interest include those encoding hemoglobin, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-4, interleukin-5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, etc., GM-CSF, G-CSF, M-CSR, human growth factor, insulin, factor VIII, factor IX, tPA, LDL receptors, tumor necrosis factor, PDGF, EGF, NGF, IL-1ra, EPO, .beta.-globin and the like, as well as biologically active muteins of these proteins. The nucleic acid utilized may also be "anti-sense" DNA or RNA, which binds to DNA or RNA and blocks the production of harmful molecules. In addition, the DNA carried to the arterial cells in accordance with the present invention may code for polypeptides which prevent the replication of harmful viruses or block the production of smooth muscle cells in arterial walls to prevent restenosis.

Antisense RNA molecules are known to be useful for regulating translation within the cell. Antisense RNA molecules can be produced from the corresponding gene sequences. The antisense molecules can be used as a therapeutic to regulate gene expression associated with a particular disease.

The antisense molecules are obtained from a nucleotide sequence by reversing the orientation of the coding region with regard to the promoter. Thus, the antisense RNA is complementary to the corresponding mRNA. For a review of antisense design see Green, et al., Ann. Rev. Biochem. 55:569-597 (1986), which is hereby incorporated by reference. The antisense sequences can contain modified sugar phosphate backbones to increase stability and make them less sensitive to RNase activity. Examples of the modifications are described by Rossi, et al., Pharmacol, Ther. 50(2):245-354, (1991).

In certain therapeutic applications, such as in the treatment of ischemic diseases, it may be desirable to induce angiogenesis, i.e., the formation of new blood vessels. For such applications, DNA's encoding growth factors, polypeptides or proteins, capable of inducing angiogenesis are selected. Folkman, et

al., Science, 235:442-447 (1987). These include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor .alpha. and .beta. (TGF-.beta. and TGF-.beta.), platelet-derived endothelial cell growth factor (PD-ECGF), platelet-derived growth factor (PDGF) itself, tumor necrosis factor .alpha. (TNF-.alpha.), hepatocyte growth factor (HGF) and insulin like growth factor. See, Klagsbrun, et al., Annu. Rev. Physiol., 53:217-239 (1991) and Folkman, et al., J. Biol. Chem. 267:10931-10934 (1992). Muteins or fragments of an angiogenic protein may be used as long as they induce or promote the formation of new blood vessels.

Recent investigations have established the feasibility of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral artery development in animal models of myocardial and hindlimb ischemia. See, Baffour, et al., J. Vasc. Surg., 16:181-191 (1992) (bFGF); Pu, et al, Circulation, 88:208-215 (1993) (aFGF); Yanagisawa-Miwa, et al., Science, 257:1401-1403 (1992) (bFGF); Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) (VEGF).

VEGF was also purified independently as a tumor-secreted factor that included vascular permeability by the Miles assay (Keck, et al, Science, 246:1309-1342 (1989) and Connolly, et al., J. Biol Chem., 264:20017-20024 (1989)), and thus its alternate designation, vascular permeability factor (VPF). VEGF is a preferred angiogenic protein. Two features distinguish VEGF from other heparin-binding, angiogenic growth factors. First, the NH.sub.2 terminus of VEGF is preceded by a typical signal sequence; therefore, unlike bFGF, VEGF can be secreted by intact cells. Second, its high-affinity binding sites, shown to include the tyrosine kinase receptors Flt-1 and Flt-1/KDR are present on endothelial cells. Ferrara, et al., Biochem. Biophys. Res. Commun., 161:851-855 (1989) and Conn, et al., Proc. Natl. Acad. Sci. USA, 87:1323-1327 (1990). (Interaction of VEGF with lower affinity binding sites has been shown to induce mononuclear phagocyte chemotaxis). Shen, et al., Blood, 81:2767-2773 (1993) and Clauss, et al., J. Exp. Med., 172:1535-1545 (1990).

Evidence that VEGF stimulates angiogenesis in vivo had been developed in experiments performed on rat and rabbit cornea (Levy, et al., Growth Factors, 2:9-19 (1989) and Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989)), the chorioallantoic membrane (Ferrara, et al., Biochem Biophys Res Commun., 161:851-855 (1989)), and the rabbit bone graft model. Connolly, et al., J. Clin. Invest., 84:1470-1478 (1989).

Preferably, the angiogenic protein contains a secretory signal sequence that facilitates secretion of the protein from the arterial cell. Angiogenic proteins having native signal sequences, e.g., VEGF, are preferred. Angiogenic proteins that do not have native signal sequences, e.g., bFGF, can be modified to contain such sequences using routine genetic manipulation techniques. See, Nabel et al., Nature 362:844 (1993).

The nucleotide sequence of numerous peptides and proteins, including angiogenic proteins, are readily available through a number of computer data bases, for example, GenBank, EMBL and Swiss-Prot. Using this information, a DNA segment encoding the desired may be chemically synthesized or, alternatively, such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

To simplify the manipulation and handling of the DNA, prior to introduction to the arterial cell, the DNA is preferably inserted into a vector, e.g., a plasmid vector such as pUC118, pBR322, or other known plasmid vectors, that includes, for example, an E. Coli origin of replication. See, Sambrook, et al., Molecular Cloning:A Laboratory Manual, Cold Spring Harbor Laboratory press, (1989). The plasmid vector may also include a selectable marker such as the .beta.-lactamase gene for ampicillin

resistance, provided that the marker polypeptide does not adversely effect the metabolism of the organism being treated. Additionally, if necessary, the DNA may be operably linked to a promoter/enhancer region capable of driving expression of the protein in the arterial cell. An example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. Normally, an enhancer is not necessary when the CMV promoter is used. The RSV and MMT promoters may also be used. Certain proteins can expressed using their native promoter.

If desired, the DNA may be used with a microdelivery vehicle such as cationic liposomes and adenoviral vectors. For a review of the procedures for liposome preparation, targeting and delivery of contents, see Mannino and Gould-Fogerite, Bio Techniques, 6:682 (1988). See also, Felgner and Holm, Bethesda Res. Lab. Focus, 11(2):21 (1989) and Maurer, R. A., Bethesda Res. Lab. Focus, 11 (2):25 (1989). Replication-defective recombinant adenoviral vectors, can be produced in accordance with known techniques. See, Quantin, et al., Proc. Natl. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricadet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992).

In certain situations, it may be desirable to use DNA's encoding two or more different proteins in order optimize the therapeutic outcome. For example, DNA encoding two angiogenic proteins, e.g., VEGF and bFGF, can be used, and provides an improvement over the use of bFGF alone. Or an angiogenic factor can be combined with other genes or their encoded gene products to enhance the activity of targeted cells, while simultaneously inducing angiogenesis, including, for example, nitric oxide synthase, L-argine, fibronectin, urokinase, plasminogen activator and heparin.

The hydrophilic polymer is selected to allow incorporation of the DNA to be delivered to the arterial cell and its release when the hydrophilic polymer contacts the arterial cell.

Preferably, the hydrophilic polymer is a hydrogel polymer, a cross-linked polymer material formed from the combination of a colloid and water. Cross-linking reduces solubility and produces a jelly-like polymer that is characterized by the ability to swell and absorb liquid, e.g., that containing the DNA. Suitable hydrogel polymers include, for example, those selected from the group consisting of polycarboxylic acids, cellulosic polymers, gelatin, polyvinylpyrrolidone, maleic anhydride polymers, polyamides, polyvinyl alcohols, and polyethylene oxides. Preferred hydrogels are polyacrylic acid polymers available as HYDROPLUS (Mansfield Boston Scientific Corp., Watertown, Mass.) and described in U.S. Pat. No. 5,091,205.

The nucleic acid in aqueous solution is incorporated into the hydrophilic polymer to form a nucleic acid-hydrophilic polymer composition. The nucleic acid is incorporated without complexing or chemical reaction with the hydrophilic polymer, and is preferably relatively freely released therefrom when placed in contact with the arterial cells. The resulting structure comprises a support, e.g. the balloon of the balloon catheter, on which is mounted the hydrogel, in or on which is incorporated the desired DNA and its associated vehicle, e.g., phage or plasmid vector. The hydrophilic polymer is preferably adhered to the support, so that after application of the DNA to the target cells, the hydrophilic polymer is removed with the support.

An arterial cell is contacted with the nucleic acid-hydrophilic polymer composition by any means familiar to the skilled artisan. The preferred means is a balloon catheter having the hydrophilic polymer on its outer surface, which permits the contact between the hydrophilic polymer bearing the nucleic acid to be transferred and the arterial cells to be made with some pressure, thus facilitating the transfer of the nucleic acid to the cells. However, other supports for the hydrophilic polymer are also useful, such as catheters or solid rods having a surface of hydrophilic polymer. Preferably, the catheters or

rods or other substrates which are flexible, to facilitate threading through the arteries to reach the point of intended application. For cells that are not in tubular arteries, other types of catheters, rods or needles may be used.

When a hydrophillic arterial balloon is used, it is not necessary to protect the balloon prior to inflation, since relatively little of the nucleic acid is lost in transit to the treatment site until the balloon is inflated and the hydrophilic polymer bearing the nucleic acid is pressed against the arterial cells. When hydrophilic polymer-surfaced catheters or rods are used as the vehicle or substrate, the surface can protected, e.g. by a sheath, until the point of intended application is reached, and then the protection removed to permit the hydrophilic polymer bearing the nucleic acid to contact the arterial cells.

The vehicle, be it arterial balloon, catheter, flexible rod or other shaped vehicle, can be furnished with means to assist in accurate placement within the intended body cavity. For example, it can be furnished with a radioactive element, or made radio-opaque, furnished with means permitting easy location using ultrasound, etc.

Preferably, the nucleic acid-hydrophilic composition contacts the arterial cell by means of a catheter. The catheter is preferably a balloon catheter constructed for insertion in a blood vessel and has a catheter shaft and an expandable dilation balloon mounted on the catheter shaft. At least a portion of the exterior surface of the expandable portion is defined by a coating of a tenaciously adhered hydrophilic. Incorporated in the hydrophilic polymer is an aqueous solution of the DNA to be delivered to the arterial cells.

In general, when dry, the hydrophilic polymer (preferably hydrogel) coating is preferably on the order of about 1 to 10 microns thick, with a 2 to 5 micron coating typical. Very thin hydrogel coatings, e.g., of about 0.2-0.3 microns (dry) and much thicker hydrogel coatings, e.g., more than 10 microns (dry), are also possible. Typically, hydrogel coating thickness may swell by about a factor of 2 to 10 or more when the hydrogel coating is hydrated.

Procedures for preparing a balloon with a hydrogel coating are set forth in U.S. Pat. No. 5,304,121, the disclosure of which is incorporated herein by reference.

A representative catheter is set forth in FIG. 6. Referring to FIG. 6, 1 is the wall of the blood vessel. The figure shows the catheter body 2 held in place by the inflation of an inflation balloon 3. The balloon comprises a hydrogel coating 4 incorporating DNA 5.

In use, the DNA, for example, is applied ex vivo to the hydrophilic polymer coating of the balloon. To facilitate application, the balloon may be inflated. If necessary, the polymer may be dried with warm air and the DNA application repeated. The amount of DNA to be applied to the arterial surface depends on the purpose of the DNA and the ability of the DNA to be expressed in the arterial cells. Generally, the amount of naked DNA applied to the balloon catheter is between about 0.1 and 100 .mu.g/mm.sup.2, more preferably between about 0.5 and about 20 .mu.g/mm.sup.2, most preferably between about 1.5 and about 8 .mu.g/mm.sup.2. Preferably, between 0.5 mg and 5 mg of DNA are applied to the hydrogel coating of a balloon catheter having an inflated lateral area of about 630 mm.sup.2 (e.g., a balloon catheter having an inflated diameter of about 5 mm and a length of about 40 mm), providing a surface having about 0.8 to about 8 .mu.g/mm.sup.2 of DNA when the balloon is inflated and contacts the interior of the artery. More preferably, between 1 mg and 3 mg of DNA are applied to the polymer, providing a DNA loading of about 1.6 to about 4.8 .mu.g/mm.sup.2.

The catheter is inserted using standard percutaneous application techniques and directed to the desired location, e.g., an artery perfusing the target tissue. For example, in the treatment of patients with occlusive peripheral arterial disease (PAD), the balloon is directed towards an artery of the leg, e.g., lilac. Once the balloon has reached its desired location, it is inflated such that the hydrogel coating of the balloon contacts the arterial cells located on the walls of the artery and remains inflated for a time sufficient to allow transfer of the DNA encoding the angiogenic protein from the hydrogel to the arterial cells. Preferred periods of balloon inflation range from 30 seconds to 30 minutes, more preferably 1 minute to 5 minutes. Surprisingly, that is normally sufficient time to permit transfer of the DNA by the method of the present invention.

Once transferred, the DNA coding for the desired therapeutic polypeptide is expressed by the arterial cells for a period of time sufficient for treatment of the condition of interest. Because the vectors containing the DNA of interest are not normally incorporated into the genome of the cells, however, expression of the protein of interest takes place for only a limited time. Typically, the therapeutic protein is only expressed in therapeutic levels for about two days to several weeks, preferably for about 1-2 weeks. Reapplication of the DNA can be utilized to provide additional periods of expression of the therapeutic polypeptide. If desired, use of a retrovirus vector to incorporate the heterologous DNA into the genome of the arterial cells will increase the length of time during which the therapeutic polypeptide is expressed, from several weeks to indefinitely.

In one preferred application, the DNA-hydrogel polymer composition can be used to deliver a DNA encoding an angiogenic protein to an arterial cell in an artery or blood vessel perfusing the target tissue. Expression of the angiogenic protein and its secretion from the arterial cell induces angiogenesis, i.e., the formation of new blood vessels, in target tissues perfused by the artery or blood vessels, allowing for the treatment of diseases associated with vascular occlusion such as limb ischemia, ischemic cardiomyopathy, myocardial ischemia, cerebral ischemia and portal hypertension.

The present invention makes genetic treatment possible which can correct heretofore incorrectable problems.

The present invention is further illustrated by the following examples. These examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Direct Gene Transfer with Hydrogel Polymer Balloon Catheter Applied to an Angioplasty Catheter Balloon Can be Used to Effect Direct Gene Transfer to the Arterial Wall.

DNA solution was applied to the surface of an angioplasty catheter balloon with a hydrogel polymer (marketed under the mark Slider.TM. with Hydroplus.RTM. by Mansfield Boston Scientific Corp., Watertown, Mass.). The catheter was constructed with a single polyethylene balloon, 2.0 mm in diameter and 2.0 cm in length. The Hydroplus.RTM. coating consists of a hydrophilic polyacrylic acid polymer, crosslinked via an isocyanate onto the balloon to form an ultra-high molecular weight hydrogel with tight adherence to the balloon surface. The thickness of the hydrogel coating when dry measures between 3-5 .mu.m; upon exposure to an aqueous environment, the coating swells to 2-3 times its initially dry thickness. In order to apply DNA to the catheter, the balloon was inflated to 4 atm, following which 20 .mu.l of DNA solution were pipetted and distributed onto the balloon surface using a sterile pipette tip. After the balloon's hydrogel polymer was covered with a homogeneous film of DNA solution, the hydrogel was dried with warm air. The above procedure was then repeated, resulting in a total of 40 .mu.l of DNA solution applied to the balloon.

For percutaneous application, luciferase DNA concentration was 3.27 .mu.g/.mu.l. DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA).

(Attempts were made to apply DNA solution to standard uncoated balloons as well. The hydrophobic surface of the polyethylene balloon, however, made it impossible to cover the balloon with a film of DNA solution.)

To determine the total amount of DNA which is successfully absorbed onto the balloon surface, 5 hydrogel balloons were coated with 40 .mu.l DNA (2 .mu.g DNA/.mu.l) containing a small amount of .sup.35 S-labeled luciferase plasmid. (Levy, et al., Growth Factors, 2:1535-1545 (1990)). A random primed DNA labeling kit (United States Biochemical, Cleveland, Ohio) was used for the labeling reaction an unincorporated nucleotides were remove by ethanol precipitation. After the coating procedure, the catheter tip was placed in 0.5 ml water for 15 minutes at room temperature, and 1.0 ml gel solubilizer (Solvable,.TM. New England Nuclear, Boston, Mass.) for 3 hours at 50.degree. C. to dissolve the gel before the scintillation fluid was added. The amount of DNA on the balloon was calculated from the quotient: [counts per minute (cpm) in a scintillation vial containing the balloon]/[cpm in a vial containing 40 .mu.l of the same lot of labeled DNA (80 .mu.g)]. Scintillation counts were corrected for quench and chemiluminescence.

After coating hydrogel balloons with 40 .mu.l of DNA solution (containing 80 .mu.g of radiolabeled DNA), and drying the gel, the magnitude of DNA retained on the hydrogel balloon was determined by comparing the amount of radioactivity on the balloons to the amount of radioactivity in 40 .mu.l of the original radiolabeled DNA solution. Scintillation counting revealed that 97.+-.2% (n=5) of the radioactively labeled DNA remained on the hydrogel coated balloon, corresponding to 78.+-.1.5 .mu.g of luciferase DNA.

Reporter Genes

The firefly luciferase gene and the gene for nuclear-specific .beta.-galactosidase (.beta.-gal) were used as reporter genes to monitor the results of the transfection procedures. The luciferase expression vector, pRSVLUC (courtesy of Dr. Allen Brasier, Massachusetts General Hospital, Boston, Mass.), consist of a full length Photinus pyralis luciferase cDNA (pJD 204) (de Wet et al., 1987) inserted into a PGEM3-plasmid (Brasier et al. Biotechniques, 7:1116-1122 (1989)), under the control of Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. The pGSVLacZ vector contains the simian virus (SV40) large tumor nuclear location signal fused to the lacZ gene (nls .beta.-gal) (Bonnerot et al., Proc. Natl. Acad. Sci. U.S.A., 84:6795-6799 (1987)) (gift from Dr. Claire Bonnerot, Institut Pasteur, Paris, France), inserted into a pGEM1-plasmid. Nuclear staining identifies the exogenous construct designed to permit nuclear translocation, and thus distinguishes expression of the transgene from endogenous (cytoplasmic) .beta.-gal activity. Previous concerns (Lim et al., Circulation, 83:2007-2011 (1991)) regarding nonspecificity of blue staining resulting from .beta.-gal are thus eliminated.

Analysis of Luciferase Activity

The magnitude of gene expression was determined by measuring luciferase activity as described previously (Leclerc et al., J. Clin. Invest., 90:936-944 (1992)) using the Luciferase Assay System (Promega, Madison, Wis.). Briefly, frozen arteries were homogenized and dissolved in 300 .mu.l of Cell Culture Lysis Reagent (Promega) containing 1 mg/ml bovine serum albumin. Three different 20-.mu.l aliquots prepared from each transfected specimen were mixed in a sample tube with 100 .mu.l of

Luciferase Assay Reagent (Promega, Madison, Wis.) and inserted into a luminometer (Model 20e, Turner Design, Sunnyvale, Calif.) that reports results on a scale established to yield as low as 10.sup.-3 Turner light units (TLU). The specimen's total luciferase activity was calculated from the mean of the three aliquots analyzed. The luciferase values were in the linear range of a standard curve derived from samples with a known amount of luciferase (Sigma, St. Louis, Mo., catalogue #L9009). The lyophilized luciferase was, according to the manufacturer's instructions, dissolved in sterile water and further diluted in Cell Culture Lysis Reagent with 1 mg/ml bovine serum albumin. The following equation was used to convert TLU into pg luciferase: Luciferase [pg]=-0.08+0.051 TLU. Using this formula, 100 TLU corresponds to 5.0 pg of luciferase. It must be noted that the specific activity of luciferase standards from different vendors can vary considerably (Wolff, et al., Biotechniques, 11:474-485 (1991)); therefore, direct comparisons of luciferase reported by different groups must be made with caution, especially when the origin of the standard used is not specified.

Percutaneous Transfection

Percutaneous gene transfer experiments with the luciferase gene were performed in 13 rabbits using a catheter with a balloon to which a 20 .mu.m hydrogel coating had been applied and which was advanced through a 5 F teflon sheath. The balloon was advanced beyond the distal tip of the sheath, coated with 130 .mu.g luciferase DNA, and pulled back into the sheath to protect the balloon from subsequent contact with blood. The sheath and the angioplasty catheter were then introduced via the right carotid artery and advanced to the left common lilac artery under fluoroscopic control. The balloon catheter was advanced 2 cm further (beyond the distal sheath tip) into the external lilac artery and inflated there for 1 or 5 min. Following balloon deflation, the catheter system was removed. In 10 animals, the transfected external lilac artery as well as the contralateral control artery were removed 3 days later, weighed, and assayed for luciferase activity. In 3 additional animals, which had been transfected for 5 min. only, the arteries were excised 14 days after gene transfer. In these 3 animals we also removed the left femoral artery to check for luciferase expression directly downstream of the transfected segment.

Results

Luciferase expression was detected in all 10 (100%) percutaneously transfected arteries excised after 3 days, whether inflated for 5 min (386.+-.299 TLU, n=5) or 1 min (113.+-.59 TLU, n=5).

Three additional animals, in which balloons were inflated for 5 min only, were sacrificed after 14 days. Individual luciferase expression was 152.6, and 16 TLU, respectively (mean=58.+-.47 TLU). In this series, we also measured luciferase in the adjacent femoral artery, which was not inflated. Luciferase expression in all these arteries was undistinguishable from background activity (mean 0.04.+-.0.29 TLU).

The findings demonstrate that endoluminal vascular gene transfer can be achieved successfully and consistently with pure DNA applied to a standard angioplasty catheter balloon coated with hydrogel polymer. The hydrogel provides the absorbable medium to which one may apply a solution of pure DNA. Drying of the gel results in a layer of concentrated DNA which is then transferred to the arterial wall as the balloon contacts the arterial wall coincident with balloon inflation. Experiments with radiolabeled DNA established that 97% of DNA applied in aqueous solution to the hydrogel-coated balloon was still present on the balloon after drying of the gel. Autoradiograms of the arterial wall demonstrated that inflation of the hydrogel balloon results in DNA uptake which is distributed across the full thickness of the arterial wall. DNA was shown to penetrate the intact internal elastic lamina and was distributed intracellularly as well as extracellularly.

Despite elimination of accessory transfection vehicles in this example, both the frequency of successful transfection and the magnitude of reporter gene expression achieved were superior to that previously reported from our laboratory (Leclerc, et al., J. Clin Invest., 90:936-944 (1992)) and comparable to the results achieved by others (Chapman, et al., Circ. Res., 71:27-33 (1992) and Lim, et al., Circulation, 83:2007-2011 (1991)) using alternative delivery schemes. The success rate of transfection in our rabbit model as measured by expression of the luciferase transgene was 100% (37 of 37 artery segments), even in those cases in which the inflation time was reduced to one minute. The duration of inflation within a range from 10 to 30 minutes did not have significant impact on transfection efficiency, a feature which would be expected to facilitate human arterial, particularly coronary, gene transfer.

EXAMPLE 2

Induction of Angiogeneses In Vivo

Methods

Animal Model (FIG. 1).

The angiogenic response to transfection of the gene for vascular endothelial growth factor (VEGF) was investigated using a rabbit ischemic hindlimb model. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994) and Pu, et al., J. Invest Surg , (In Press). All protocols were approved by St. Elizabeth's Institutional Animal Care and Use Committee. Male New Zealand White rabbits weighing 4-4.5 kg (Pine Acre Rabbitry, Norton, Mass.) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) following premedication with xyazine (2.5 mg/kg). A longitudinal incision was then performed, extending inferiorly from the inguinal ligament to a point just proximal to the patella. The limb in which the incision was performed--right versus left--was determined at random at the time of surgery by the surgeon. Through this incision, using surgical loops, the femoral artery was dissected free along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex and superficial epigastric arteries, were also dissected free. After further dissecting the popliteal and saphenous arteries distally, the external lilac artery as well as all of the above arteries were ligated. Finally, the femoral artery was completely excised from its proximal origin as a branch of the external lilac artery, to the point distally where it bifurcates into the saphenous and popliteal arteries. Once the femoral artery is excised, thrombotic occlusion of the external lilac artery extends retrograde to its origin from the common lilac (FIG. 1(a), arrow). As a result, the blood supply to the distal limb is dependent on the collateral arteries which may originate from the internal lilac artery. Accordingly, direct arterial gene transfer of VEGF was performed in to the internal lilac artery of the ischemic limb. Post-operatively, all animals were closely monitored. Analgesia (levorphanol tartrate 60 mg/kg, Roche Laboratories, Nutley, N.J.) was administered subcutaneously as required for evidence of discomfort throughout the duration of the experiment. Prophylactic antibiotics (enrofloxacin 2.5 mg/kg, Miles, Shawnee Mission, Kans.) was also administered subcutaneously for a total of 5 days post-operatively.

Plasmids and Smooth Muscle Cell (SMC) Transfection in Vitro.

Complementary DNA clones for recombinant human VEGF.sub.165, isolated from cDNA libraries prepared from HL60 leukemia cells, were assembled into a mammalian expression vector containing the cytomegalovirus promoter. Leung, et al., Science, 246:1306-1309 (1989). The biological activity of VEGF.sub.165 secreted from cells transfected with this construct (phVEGF.sub.165) was

previously confirmed by the evidence that media conditioned by transfected human 293 cells promoted the proliferation of capillary cells. Leung, et al., Science, 246:1306-1309 (1989).

To evaluate expression of phVEGF.sub.165 in vascular cells, rabbit arterial smooth muscle cells (SMCs) were transfected in vitro. Cells were cultured by explant outgrowth from the thoracic aorta of New Zealand White rabbits. The identity of vascular SMCs was confirmed morphologically using phase contrast microscopy and by positive immunostaining using a monoclonal antibody to smooth muscle .alpha.-actin (Clone 1A4, Sigma, St. Louis, Mo.). Cells were grown in the media (M199, GIBCO BRL, Gaithersberg, Md.) supplemented with 10% fetal bovine serum (FBD, GIBCO BRL). In vitro transfection was performed by incubating SMCs (1.48.times.10.sup.6 cells/10 cm plate) with 11.5 .mu.g of the plasmid DNA and 70 .mu.g of liposomes (Transfection-reagent, Boehringer Mannheim, Indianapolis, Ind.) as previously described. Pickering, et al., Circulation, 89:13-21 (1994). After completion of transfection, media was changed to 10% FBS. Culture supernatant was sampled at 3 days pot-transfection, and was analyzed by ELISA assay for VEGF protein. Houck, et al., J. Biol. Chem 267:26031-26037 (1992).

The plasmid pGSVLacZ (courtesy of Dr. Claire Bonnerot) containing a nuclear targeted .beta.-galactosidase sequence coupled to the simian virus 40 early promoter (Bonnerot, et al., Proc. Natl. Acad. Sci. USA, 84:6795-6799 (1987)) was used for all the control transfection experiments.

Percutaneous Arterial Gene Transfer in Vitro.

An interval of 10 days between the time of surgery and gene transfer was allowed for post-operative recovery of rabbits and development of endogenous collateral vessels. Beyond this time-point, studies performed up to 90 days post-operatively (Pu, et al., J. Invest. Surg., (In Press)) have demonstrated no significant collateral vessel augmentation. At 10 days post-operatively (day 0), after performing a baseline angiogram (see below), the internal lilac artery of the ischemic limb of 8 animals was transferred with phVEGF.sub.165 percutaneously using a 2.0 mm hydrogel-coated balloon catheter (Slider.TM. with HYDROPLUS,.RTM. Boston Scientific, Watertown, Mass.). The angioplasty balloon was prepared (ex vivo) by first advancing the deflated balloon through a 5 Fr. teflon sheath (Boston Scientific), applying 400 .mu.g of phVEGF.sub.165 to the 20 .mu.m-thick later of hydrogel on the external surface of the inflated balloon, and then retracting the inflated balloon back into the protective sheath. The Sheath and angioplasty catheter were then introduced via the right carotid artery, and advanced to the lower abdominal aorta using a 0.014 inch guidewire (Hi-Torque Floppy II, Advanced Cardiovascular Systems, Temecula, Calif.) under fluoroscopic guidance. The balloon catheter was then advanced out of the sheath into the internal lilac artery of the ischemic limb, inflated for 1 min at 6 atmospheres, deflated, and withdrawn (FIG. 1(a), open arrow). An identical protocol was employed to transfect the internal iliac artery of 9 control animals with the plasmid pGSVLacZ containing a nuclear targeted .beta.-galactosidase sequence. Heparin was not administered at the time of transfection or angiography.

Evaluation of Angiogenesis in the Ischemic Limb.

Development of collateral vessels in the ischemic limb was serially evaluated by calf blood pressure measurement and internal lilac arteriography immediately prior to transfection (day 0), and then in serial fashion at days 10 and 30 post-transfection. On each occasion, it was necessary to lightly anesthetize the animal with a mixture of Ketamine (10 mg/kg) and acepromazine (0.16 mg/kg) following premedication with xyazine (2.5 mg/kg). Following the final 30-day follow-up, the animal was sacrificed, and tissue sections were prepared from the hindlimb muscles in order to perform analysis of capillary density. These analyses are discussed in detail below.

Calf Blood Pressure Ratio.

Calf blood pressure was measured in both hindlimbs using a Doppler Flowmeter (Model 1050, Parks Medical Electronics, Aloha, Oreg.), immediately prior to transfection (day 0), as well as on days 10 and 30. On each occasion, the hindlimbs were shaved and cleaned; the pulse of the posterior tibial artery was identified using a Doppler probe; and the systolic pressure of both limbs was determined using standard techniques. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to systolic pressure of the normal limb.

Selective Internal Iliac Arteriography.

Collateral artery development in this ischemic hindlimb model originates from the internal lilac artery. Accordingly, selective internal lilac arteriography was performed on day 0 (immediately prior to transfection), and again on days 10 and 30 post-transfection as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A 3 Fr. end-hole infusion catheter (Tracker-18, Target Therapeutics, San Jose, Calif.) was introduced into the right common carotid artery through a small cutdown, and advanced to the internal lilac artery at the level of the interspace between the seventh lumbar and the first sacral vertebrae. Following intra-arterial injection of nitroglycerin (0.25 mg, SoloPak Laboratories, Franklin Park, Ill.), a total of 5 ml of contrast media (Isovue-370, Squibb Diagnostics, New Brunswick, N.J.) was then injected using an automated angiographic injector (Medrad, Pittsburgh, Pa.) programmed to reproducibly deliver a flow rate of 1 ml per sec. Serial images of the ischemic hindlimb were then recorded on 105-mm spot film at a rate of 1 film per sec for at least 10 sec. Following completion of arteriography, the catheter was removed and the wound was closed. All of the above-described procedures were completed without the use of heparin.

Morphometric angiographic analysis of collateral vessel development was performed as previously described. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). A composite of 5-mm.sup.2 grids was placed over the medical thigh area of the 4-sec angiogram. The total number of grid intersections in the medical thigh area, as well as the total number of intersections crossed by a contrast-opacified artery were counted individually by a single observer blinded to the treatment regimen. An angiographic score was calculated for each film as the ratio of grid intersections in the medial thigh.

Capillary Density and Capillary/Myocyte Ratio.

The effect of VEGF gene transfer upon anatomic evidence of collateral artery formation was further examined by measuring the number of capillaries in light microscopic sections taken from the ischemic hindlimbs. Takeshita, et al., J. Clin. Invest., 93:662-670 (1994). Tissue specimens were obtained as transverse sections from the ischemic limb muscles at the time of sacrifice (day 30 post-transfection). Muscle samples were embedded in O.C.T. compound, (Miles, Elkhart, Ind.) and snap-frozen in liquid nitrogen. Multiple frozen sections (5 .mu.m in thickness) were then cut from each specimen on a cryostat (Miles), so that the muscle fibers were oriented in a transverse fashion, and two sections then placed on glass slides. Tissue sections were stained for alkaline phosphate using an indoxyl-tetrazolium method to detect capillary endothelial cells (Ziada, et al., Cardiovasc. Res., 18:724-732 (1984)), and were then counterstained with eosin. Capillaries were counted under a 20x objective to determine the capillary density (mean number of capillaries per mm.sup.2). A total of 20 different fields was randomly selected, and the number of capillaries counted. To ensure that analysis of capillary density was not overestimated due to muscle atrophy, or underestimated due to interstitial edema, capillaries identified at necropsy were also evaluated as a function of myocytes in the

histologic section. The counting scheme used to compute the capillary/myocyte ratio was otherwise identical to that used to compute capillary density.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Southern Blot Analysis, and Sequencing of RT-PCR Product.

The presence of human VEGF mRNA was detecting using RT-PCR. Arterial samples were obtained at 5 days post-transfection, and total cellular RNA was isolated using TRI REAGENT (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. Extracted RNA was treated with DNase I (0.5 .mu.l, 10 U/.mu.l, RNase-free, Message Clean kit, GenHunter, Boston, Mass.) at 37.degree. C. for 30 min to eliminate DNA contamination. The yield of extracted RNA was determined spectrophotometrically by ultraviolet absorbance at 260 nm. To check that the RNA was not degraded and electrophoresed through a 1% non-denaturing miniagarose gel. 0.5 .mu.g of each RNA sample was used to make cDNA in a reaction volume of 20 .mu.l containing 0.5 mM of each deoxynucleotide triphosphate (Pharmacia, Piscataway, N.J.), 10 mM dithiothreitol, 10 units of RNasin (Promega, Madison, Wis.), 50 mM Tris-HCI (pH 8.3), 75 mM KCL, 3 mM MgCl.sub.2, 1 .mu.g random hexanucleotide primers (Promega), and 200 units of M-MLV reverse transcriptase (GIBCO BRL). For greater accuracy and reproducibility, master mixes for a number of reactions were made up and aliquoted to tubes containing RNA. Reactions were incubated at 42.degree. C. for 1 hr, then at 95.degree. C. for 5 min to terminate the reaction. Twenty .mu.l of diethyl pyrocarbonate (DEPC) water was then added and 5 .mu.l of the diluted reaction (1/8th) was used on the PCR analysis. The optimized reaction in a total volume of 20 .mu.l contained 0.2 mM of each deoxynucleotide triphosphate, 3 mM MgCl.sub.2, 2 .mu.l PCR II buffer (Perkin-Elmer, Norwalk, Conn.; final concentrations, 50 mM KCL, 10 mM Tris-HCL), 5 ng/.mu.l (13.77 pmoles) of each primer, and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR was performed on a 9600 PCR system (Perkin-Elmer) using microamp 0.2. ml thin-walled tubes. Amplification was for 40-45 cycles of 94.degree. C. for 20 sec, 55.degree. C. for 20 sec, and 72.degree. C. for 20 sec, ending with 5 min at 72.degree. C. To test for false positives, controls were included with no RNA and no reverse transcriptase. A pair of oligonucleotide primers (22 mers) was designed to amplify a 258 bp sequence from the mRNA of human VEGF. To ensure specificity and avoid amplification of endogenous rabbit VEGF, each primer was selected from a region which is not conserved among different species. Sequences of primers used were: 5'-GAGGGCAGAATCATCACGAAGT-3' (sense) SEQ. ID NO:1 ; 5'-TCCTATGTGCTGGCCTTGGTGA-3' (antisense) SEQ. ID NO:2. RT-PCR products were transferred from agarose gels to nylon membranes (Hybond, Amersham, Arlington Heights, Ill.). The probe was 5' end-labelled with T4 polynucleotide kinase and [.beta.-.sup.32 P]ATP (Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1989)) and hybridized to the nylon filters using Rapid Hybridization buffer (Amersham) according to manufacturer's instructions. To visualize hybridized bands, filters were exposed to X-ray film (Kodak Xar-5).

To confirm the identity of VEGF PCR products. DNA bands were excised from agarose gels, purified using Geneclean (BIO 101, La Jolla, Calif.), and sequenced directly (i.e. without subcloning) using dsDNA Cycle Sequencing System (GIBCO BRL) following the directions of manufacturer. The two VEGF primers used for PCR were 5' end-labeled with [.beta.-.sup.32 P]ATP and T.sub.4 polynucleotide kinase and used as sequencing primers to determine the sequence of both strands of the PCR product.

.beta.-Galactosidase Staining of Transfected lilac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, .beta.-galactosidase activity was determined

by incubation of arterial segments with 5-bromo-4-chloro-3-indolyl .beta.-D-galactosidase chromogen (X-Gall), Sigma) as previously described. Riessen, et al., Hum. Gene Ther., 4:749-758 (1993). Following staining with X-Gal solution, tissues were paraffin-embedded, sectioned, and counterstained with nuclear fast red. Nuclear localized .beta.-galactosidase expression of the plasmid pGSVLacZ cannot result from endogenous .beta.-galactosidase activity; accordingly, histochemical identification of .beta.-galactosidase within the cell nucleus was interpreted as evidence for successful gene transfer and gene expression. Cytoplasmic or other staining was considered non-specific for the purpose of the present study.

Statistics

Results were expressed as means.+-.standard deviation (SD). Statistical significance was evaluated using unpaired Student's t test for more than two means. A value of $p<0.05$ was interpreted to denote statistical significance.

Results

ELISA Assay for VEGF. To test the expression of the plasmid phVEGF.sub.165 in vascular cells, culture supernatant of VEGF-transfected SMCs (1.48.times.10.sup.6 cells/10 cm plate) was sampled at 3 days post-transfection, and analyzed by ELSA for VEGF protein. The media of VEGF-transfected SMCs contained an average of 1.5 .mu.g of VEGF protein (n=3). In contrast, culture media of .beta.-galactosidase-transfected SMCs (n=3) or non-transfected SMCs (n=3) did not contain detectable levels of VEGF protein.

RT-PCR, Southern Blot Analysis, and Sequencing of RT-PCR Product.

To confirm expression of human VEGF gene in transfected rabbit lilac arteries in vivo, we analyzed transfected arteries for the presence of human VEGF mRNA by RT-PCR. As indicated above, to ensure the specificity of RT-PCR for human VEGF mRNA resulting from successful transfection (versus endogenous rabbit VEGF mRNA), primers employed were selected from a region which is not conserved among different species. Arteries were harvested at 5 days post-transfection. The presence of human VEGF mRNA was readily detected in rabbit SMC culture (n=3) and rabbit lilac arteries (n=3) transfected with phVEGF.sub.165. Rabbit lilac arteries transfected with pGSVLacZ (n=3) were negative for human VEGF mRNA (FIG. 2(a)). Southern blot analysis was used to further confirm that the 158 bp bands obtained by RT-PCR did in fact correspond to the region between the two primers (FIG. 2(b)). Direct sequencing of the RT-PCR product document that this band represented the human VEGF sequence (FIG. 2(c)).

Angiographic Assessment.

The development of collateral vessels in the 5 rabbits transfected with phVEGF.sub.165 and 6 rabbits transfected with pGSVLacZ was evaluated by selective internal lilac angiography. FIG. 3 illustrates representative internal lilac angiogram recorded from both control and VEGF-transfected animals. In control animals, collateral artery development in the medial thigh typically appeared unchanged or progressed only slightly in serial angiogram recorded at days 0, 10, and 30 (FIGS. 3(a-c)). In contrast, in the VEGF-transfected group, marked progression of collateral artery was observed between days 10 and 30 (FIGS. 3, (d-f)). Morphometric analysis of collateral vessel development in the media thigh was performed by calculating the angiographic score as described above. At baseline (day 0), there was no significant difference in angiographic score between the VEGF-transfected and control groups (day 0:0.17.+-.0.02 vs 0.20.+-.0.06, p=ns). By day 30, however, the angiographic score in VEGF-

transfected group was significantly higher that in control group (0.47.+-.0.09 vs 0.34.+-.0.10, p<0.05) (FIG. 4(a)).

Calf Blood Pressure Ratio (FIG. 4(b)).

Reduction of the hemodynamic deficit in the ischemic limb following VEGF-transfection was confirmed by measurement of calf blood pressure ratio (ischemic/normal limb). The calf blood pressure ratio was virtually identical in both groups prior to transfection (0.23.+-.0.12 in VEGF-transfected animals, p=ns). By day 10 post-transfection, the blood pressure ration for VEGF-transfected rabbits was significantly higher than for the control rabbits (0.60.+-.0.12 vs 0.32.+-.0.14, p<0.01). At day 30, the blood pressure ratio for the VEGF-transfected group continued to exceed that of controls (0.70.+-.0.08 vs 0.50.+-.0.18, p<0.05).

Capillary Density and Capillary/Myocyte Ratio (FIGS. 4(c), 5).

A favorable effect of VEGF-transfection upon revascularization was also apparent at the capillary level. The medial thigh muscles of the ischemic limbs were histologically examined at day 30 post-transfection. Analysis of capillary density disclosed a value of 233.0.+-.60.9/mm.sup.2 in VEGF-transfected group versus 168.7.+-.31.5/mm.sup.2 in the control group (p<0.05). Analysis of capillary/myocyte ratio disclosed a value of 0.67.+-.0.15 in the VEGF-transfected group versus 0.48.+-.0.10 in the control group (p<0.05).

.beta.-Galactosidase Staining of Transfected Iliac Arteries.

To evaluate the efficiency of in vivo arterial gene transfer, transfected lilac arteries were harvested at 5 days post-transfection, and were used for .beta.-galactosidase histochemical analysis. In arteries transfected with nuclear targeted .beta.-galactosidase, evidence of successful transfection, indicated by dark blue nuclear staining, was observed in only<0.5% of total arterial cells. Arteries transfected with phVEGF.sub.165 were negative for nuclear staining.

EXAMPLE 3

Comparison of Double-Balloon Catheter Technique and Hydrogel-Coated Balloon Catheter Technique

Methods

Recombinant Adenoviral Vectors

Replication-defective recombinant adenoviral vectors, based on human adenovirus 5 serotype, were produced as previously described. Quantin, et al., Proc. Nat. Acad. Sci. USA, 89:2581-2584 (1992); Stratford-Perricaudet, et al., J. Clin. Invest., 90:626-630 (1992); and Rosenfeld, et al., Cell, 68:143-155 (1992). Ad-RSV.beta.gal contains the Escherichia coil lac Z gene and the SV40 early region nuclear localization sequence (nls). The nlslacZ gene encodes a nuclear-targeted .beta.-galactosidase under the control of the Rous sarcoma virus promoter. Ad-RSVmDys, used as a negative control, contains a human "minidystrophin" cDNA under the control of the same promoter. Ragot, et al., Nature, 361:647-650 (1993).

In Vivo Percutaneous Gene Transfer Procedures

All animal procedures were approved by the Institutional Animal Care and Use Committees of Faculte Bichat and St. Elizabeth's Hospital. Gene transfer was performed in the external lilac artery of 29 New Zealand white rabbits under general anesthesia and sterile conditions. Anesthesia was induced with intramuscular acepromazine and maintained with intravenous pentobarbital. Adenoviral stocks were used within 30 minutes of thawing.

1. Double-balloon catheter technique.

In 15 animals, Ad-RSV.beta.gal (2.10.sup.9 to 2.10.sup.10 plaque forming unites {pfu} in 2 ml PBS) was transferred to the right lilac artery, either normal (n=9) or previously denuded (n=6), using a 4 French double-balloon catheter (Mansfield Medical, Boston Scientific Corp., Watertown, Mass.) as previously described. Nabel, et al., Science, 244:1342-1344 (1989). The catheter was positioned in a segment of the artery which lacked angiographically visible side branches. The viral solution was maintained in contact with the arterial wall for 30 min. The left lilac artery of the same 15 animals was used as a control: in 7 animals no catheter was inserted, in 6 animals the endothelium was removed using balloon abrasion, and, in the 2 other animals, a double-balloon catheter was used in infuse Ad-RSVmDys (2.10.sup.9 pfu in 2 ml PBS).

2. Hydrogel-Coated Balloon Catheter Technique.

In 14 animals, a hydrogel-coated balloon catheter was used (Slider.TM. with Hydroplus.RTM., Mansfield Medical, Boston Scientific Corp., Watertown, Mass.). The balloon diameter (either 2.5 or 3.0 mm), was chosen to approximate a 1.0 balloon/artery ratio based on caliper measurement of magnified angiographic frames. Ad-RSV.beta.gal (1-2.10.sup.10 pfu in 100 .mu.l PBS) was applied to the polymer-coated balloon using a pipette as described above. The catheter was introduced into the right femoral artery through a protective sheath, the balloon was inflated at 1 atm, and the assembly was then advanced over a 0.014" guide wire to the external lilac artery where, after balloon deflation, the catheter alone was advanced 2 cm further and the balloon inflated for 30 minutes at 6 atm (ensuring nominal size of the inflated balloon). The contralateral lilac artery was in each case used as a control: in 9 animals no catheter or virus was introduced, in 2 the endothelium was removed, while in 3 a hydrogel-coated balloon catheter was used to transfer Ad-RSVmDys.

Detection of lacZ Expression in the Arterial Wall.

Three to seven days after transfection, the animals were sacrificed by pentobarbital overdose. To assess nlslacZ gene expression, the arteries were harvested and stained with X-Gal reagent (Sigma) for 6 hours, at 32.degree. C., as previously described. Sanes, et al., EMBO J., 5:3133-3142 (1986). Samples were then either mounted in OCT compound (Miles Laboratories Inc., Ill.) for cryosectioning or embedded in paraffin, cut into 6-.mu.m sections, and counterstained with hematoxylin and eosin or elastic trichrome. Expression of nlslacZ gene was considered positive only when dark blue staining of the nucleus was observed. To determine which cell types within the arterial wall expressed the transgene, immunohistochemical staining of X-Gal-stained arterial sections was performed, using a mouse monoclonal anti-.alpha.-actin primary antibody specific for vascular smooth muscle (HHF-35, Enzo Diagnostics, Farmingdale, N.Y.), and then a polyclonal peroxidase-labeled anti-mouse immunoglobulin G secondary antibody (Signet Laboratories, Dedham, Mass.).

Morphometric Analysis of nlslacZ Gene Expression in the Media.

For each transfected iliac artery, at least 2 samples were taken from the target-zone, and from each sample, at least 3 sections were examined by light microscopy after X-gal staining. Due to the

heterogeneity of .beta.-galactosidase activity on gross examination, the percentage of transfected medial cells per artery section was determined in regions showing high .beta.-galactosidase activity by counting stained versus total nuclei. The total numbers of studied medial cells were 14.10.sup.3 (n=50 sections) in the double-balloon catheter and the hydrogel-coated balloon catheter groups respectively.

Detection of Remote.sup.nlslacZ Gene Transfer and Expression.

Tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site were harvested immediately after sacrifice. For each specimen, nlslacZ gene presence and expression were assessed by polymerase chain reaction (PCR) and histochemistry (X-gal staining) respectively.

For PCR, genomic DNA was extracted from tissues by standard techniques. DNA amplification was carried out using oligodeoxynucleotide primers designed to selectively amplify Ad-RSV.beta.gal DNA over endogenous .beta.-galactosidase gene by placing one primer in the adenovirus sequence coding for protein 9 and the other primer in the lacZ sequence (5'-AGCCCGTCAGTATCGGCGGAATTC-3' (SEQ ID NO:3) and 5'-CAGCTCCTCGGTCACATCCAG-3' (SEQ ID NO:4) respectively, Genset, Paris, France). The reactions were performed in a DNA thermocycler (GeneAmp PCR System 9600, Perkin Elmer Cetus, Norwalk, Conn.) following 2 different protocols: a hold at 95.degree. C. for 3 min, 35 or 45 cycles of 95.degree. C. for 30 s, 65.degree. C. for 40 s, and 72.degree. C. for 1 min, then a final extension at 72.degree. C. for 5 min. When PCR was performed on plasmid DNA containing the nlslacZ gene used for the preparation of the adenoviral vector, or on positive liver samples obtained by deliberate systemic injection of Ad-RSV.beta.gal, the amplification reaction produced a 700 bp DNA fragment. To determine sensitivity of these procedures, DNA was extracted from liver of uninfected rabbits, aliquoted into several tubes, and spiked with dilutions of the plasmid containing the nlslacZ gene and used as a positive control. Following the amplification protocols described above, it was determined that the 35- or 45-cycle PCR could detect one copy of the nlslacZ gene in 3.102 and 3.104 cells respectively. DNA extractions and amplifications were performed simultaneously and in duplicate for studied tissues and positive controls.

Each tissue sample was also processed for histochemical analysis following the same protocol described for the arteries. For each specimen, at least 3 different segments were obtained, embedded in paraffin, and cut into at least 5 sections. Sections were counterstained with hematoxylin and eosin, and examined by light microscopy for the presence of deep blue nuclei indicative of .beta.-galactosidase expression. The number of positive cells as well as the total number of cells were counted. The total number of cells examined per sample ranged from 25.10.sup.3 to 115.10.sup.3.

Statistics

Results are expressed as mean.+-.standard deviation (SD). Comparisons between groups were performed using Student's t test for unpaired observations. A value of p<0.05 was accepted to denote statistical significance.

Results

Histological and Histochemical Analyses of Transfected Arteries Following Double-Balloon Catheter Delivery

Gross examination of all the arteries (n=15) following X-gal staining showed punctiform, heterogeneous, blue staining on the luminal aspect of the arteries, always limited to the area between

the two balloons. For the 9 normal arteries, microscopic examination disclosed dark blue nuclear staining, confined entirely to the endothelium. In contrast, when endothelial abrasion preceded transfection (n=6), X-gal staining imparted a mottled appearance to the luminal aspect of the artery. In these cases, microscopic examination showed that the endothelium had been removed and that the site of X-gal staining was subjacent to the intact internal elastic lamina, involving sparse medial cells. The identity of the transfected medial cells as smooth muscle cells was confirmed by immunohistochemical staining with monoclonal anti-.alpha.-actin antibody. Control arteries showed no nuclear blue staining.

Histological and Histochemical Analysis of Transfected Arteries Following Hydrogel-Coated Balloon Catheter Delivery

Gross examination of all the arteries after X-gal staining (n=14) showed dark blue, heterogeneous staining of the transfected site with a sharp boundary between the transfected segment and the bordering proximal and distal segments. Microscopic examination showed no residual intact endothelium; the continuity of the internal elastic lamina, in contrast, appeared preserved without apparent disruption. In the areas showing evidence of .beta.-galactosidase activity on gross examination, light microscopic examination revealed nearly continuous layers of cells with dark blue nuclear staining, generally limited to the superficial layers of the media; occasionally, sparsely distributed cells from deeper layers of the media expressed the transgene as well. Staining with monoclonal anti-.alpha.-actin antibody confirmed that transfected cells were vascular smooth muscle cells. No evidence of nuclear .beta.-galactosidase activity was seen in control arteries.

Morphometric Analysis of nlslacZ Gene Expression in the Media.

The percentage of transduced cells per artery section in regions showing high .beta.-galactosidase activity was significantly higher in the hydrogel-coated balloon catheter group than in the double-balloon catheter group (6.1.+-.2.3% vs. 0.4.+-.0.6%, p<0.0001).

Detection of Remote lacZ Gene Transfer and Expression in Other Organs

In all animals of both groups, gross and microscopic examination of X-gal stained tissue samples from liver, brain, testes, heart, lungs, kidneys, contralateral limb skeletal muscle, and arterial segments adjacent to the treated arterial site failed to show expression of nuclear-targeted .beta.-galactosidase, except in the liver of one rabbit in the double-balloon catheter group which disclosed a limited area of nuclear and peri-nuclear blue staining. In this area, less than 1/2.10.sup.3 cells expressed .beta.-galactosidase. In a few macrophages limited to samples removed from the lungs and testes of one hydrogel-coated balloon catheter treated rabbit, blue staining of the cytoplasm without nuclear staining was observed; the exclusively cytoplasmic location of .beta.-galactosidase activity in these cases, however, suggested that staining resulted from endogenous .beta.-galactosidase.

All of the above tissue samples were then screened by PCR. When the PCR was run for 35 cycles, the presence of DNA sequence specific for Ad-RSV.beta.gal was non-detectable, including in tissue samples from those animals with the highest percentage of transfected lilac arterial cells. Using an optimized protocol of 45 cycles, however, PCR was positive in the single liver that was observed to express .beta.-galactosidase, but in none of the other tissues.

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements thereon without departing from the spirit and scope of the invention as set forth in the claims.

HARVARD UNIVERSITY Gazette

The following articles appeared in the May 14, 1998, issue. Brief items have been omitted.

EXHIBIT C-6

Women In the Ivy League

Conference To Examine the Changing Nature of Journalism

Ann Blair Awarded Radcliffe Junior Faculty Fellowship at Bunting

Exhibit of German Drawings, Watercolors at Sackler Through June 7

New Arteries Grown In Diseased Hearts

By William J. Cromie

Gazette Staff

Almost anything Hugh Curtis did gave him a pain in the heart. Even when lying in bed, he felt the stabbing chest pains of angina, a hurtful signal that his heart was not getting enough oxygen.

Curtis underwent a quadruple bypass in 1986, then a single bypass late last year. Surgeons removed veins from his legs and grafted them onto his heart to bypass his blocked coronary arteries. But that didn't solve his problem.

He also received a series of angioplasties, wherein tiny balloons were threaded into his heart's arteries, then inflated. This process pushed the blockages aside, opening his arteries. Five pieces of metal mesh were installed to keep them open, but his coronary arteries closed in other places.

"I couldn't walk very far, couldn't even make my bed," says the 55-year-old resident of Danvers, Mass. "Climbing stairs was out, so was any thought of going on vacation."

Late last year, he was asked by researchers at Beth Israel Deaconess Medical Center in Boston if he wanted to volunteer for an experimental procedure at the Harvard-affiliated hospital. The procedure involved doctors injecting proteins called growth factors into his heart to stimulate growth of new blood around those clogged with plaque.

"I didn't hesitate to give them the go-ahead," Curtis recalls.

The cardiologists threaded a thin hollow tube from his groin into his heart. Through the tube they injected what is called basic fibroblast growth factor, or bFGF.

Four months after the treatment, Curtis is back working full time at a desk job in a printing company. "I no longer take 3-to-6 nitroglycerin tablets a day, and I'm painting the hallway in my house," he says cheerily. "I may never go back to playing racquetball, but I'm leading a normal life, and that's all I'm looking for."

"All his symptoms are gone," says Michael Simons, associate professor of medicine at Harvard Medical School. "He is one of 18 patients who participated in a trial of bFGF. All are now largely without

symptoms such as chest pain, shortness of breath, and fatigue."

Bypassing Bypass Surgery

Eighteen other patients who received heart-artery bypasses got bFGF at the same time. Frank Sellke, an associate professor of surgery at Harvard Medical School, implanted capsules that slowly release the drug at sites where blocked vessels were too small or too diffusely diseased to bypass.

"These patients have undergone treadmill stress tests," Simons comments. "They also have been examined with a new type of magnetic resonance imaging (MRI) that measures blood flow and detects new vessel development. It is too early to scream and shout with success, but we are pleased with the results obtained so far."

"I had an MRI a couple of weeks ago, and it showed new arteries growing and bypassing some blockage," says Curtis. "I'm getting 70 percent blood flow to an area of the heart that was down to 30 percent flow. And there's reason to think things will improve more with time."

John Modugno, 48, received bFGF in February, and his MRI tests also show evidence of new arterial growth. "I feel much better," he says, "although I'm still on drugs and get a little angina at the end of the day."

Tests of bFGF and other growth factors now under way at various research centers raise hopes that newly grown blood vessels will replace arteries choked off by atherosclerosis, thus heading off thousands, maybe millions, of heart failures and heart attacks.

If these tests continue to be successful in humans, they could lead to heart drugs that will be cheaper, safer, and a lot easier on patients than bypass surgery and angioplasty. About a million people undergo such procedures in the United States each year, but they don't always work. As in Hugh Curtis's case, some vessels are too small or located where they can't be bypassed with sections of vein. After arteries have been opened by an inflated balloon or other types of angioplasty, about one-third of them close again, some in a matter of months.

"We once thought people in which neither procedure worked accounted for only a small subgroup of patients," Simons says. "But now we're getting phone calls almost every day, so we must conclude that there are more people with this problem than we imagined."

The revolutionary potential of growth factors, of course, goes far beyond such people. Simons sees it as "having the potential to replace or reduce the use of bypass surgery." The American Heart Association estimates that 500,000 bypasses are performed each year at an average cost of $45,000 per treatment.

Severely blocked coronary arteries cause more than 3 million heart failures a year, and 7 million more people suffer the chest pains of angina. "Growth-factor treatments might be expanded to many, if not all, of these patients," Simons declares.

The Side-Effects Question

Researchers at Beth Israel Deaconess Medical Center initiated such treatments in 1996. Today, seven

teams worldwide work on growing new blood vessels with bFGF and another protein known as vascular endothelial growth factor, or VEGF (see April 23 *Gazette*, page 1).

In a trial conducted at several medical centers, VEGF was given to 17 people whose blocked coronary arteries lay out of reach of angioplasty. Fifteen of the 17 patients showed various levels of improvement.

Jeffrey Isner, a cardiologist at St. Elizabeth's Medical Center in Boston, has used VEGF to grow new vessels around blockages in the leg veins of diabetics. He has treated 30 diabetic patients, as well as five other patients with heart disease.

"Preliminary results look good in both types of disease," Isner says. "This is a very encouraging and exciting area of treatment."

The great promise of bypassing blood-vessel blockages won't become a reality, however, if the growth factors cause severe side effects.

Both bFGF and VEGF lower blood pressure. "That fact limits the amount you can give a person," Simons notes. "But that's something we can work around."

More serious is the possibility of damage to sight caused by overgrowth of blood vessels in the eye. "We have been looking carefully for this, but have not seen any as yet with bFGF," Simons comments. Also, no new blood vessels were seen growing in the eyes of patients treated with VEGF, another encouraging sign.

The most worrisome possibility concerns growth of blood vessels that might nourish small, hidden cancer tumors. Judah Folkman, another Harvard researcher, found that such tumors remain benign unless new blood vessels carry nutrients to them. Once connected to a steady blood supply, tumors grow and spread.

Folkman and Michael O'Reilly developed two exciting new cancer drugs, endostatin and angiostatin, which block rather than promote development of blood vessels.

"We hope that tumor growth can be avoided because we give the growth factor for a very short time and in small amounts," Simons notes. "It's not like we're adding a foreign substance to the body; everyone has such small amounts of bFGF circulating naturally in their bloodstream."

The side-effects issue will be addressed in tests involving larger numbers of patients. Plans call for testing both growth factors on 400 to 500 people at a combination of medical centers throughout the country. Simons expects to start expanded trials of bFGF this summer in a collaboration with Emory University in Atlanta.

A question still to be answered is exactly how new blood vessels form. The bare-bones explanation has bFGF binding to the surface of, then stimulating growth of endothelial cells, those that line the inside of capillaries, the smallest vessels. These cells leave the vessels, migrate to the tip of the capillaries, and form a tube that extends their reach.

Simons's team took startling photos of new vessels growing around blocked arteries in animals. They show small extensions sprouting like twigs on a tree limb, moving around the barricade and reconnecting on the other side.

"It's amazing to see," Simons says. "If we can continue to do the same thing in humans, without deleterious side effects, we have a chance to benefit millions of people."

END

College Admission Yield Is Nearly 80%

Highest in 25 years

Nearly 80 percent of students admitted to the Class of 2002 have chosen to enroll, the highest yield since the early 1970s, according to the Undergraduate Admissions Office. This yield is the best in more than 25 years.

Yield, the percentage of admitted candidates who decide to accept an offer of admission, is considered a measure of a school's competitiveness. Harvard's yield is again, by a wide margin, the highest of the nation's selective colleges. When the final figures are available, the yield could go even higher -- it is already well above last year's yield of 76.3 percent.

The 2,073 students admitted to the Class of 2002 were selected from a pool of 16,819 applicants. For the seventh time in eight years, applications for admission to Harvard and Radcliffe have risen. Last year, 16,597 students applied for the 1,650 places in the entering class.

The substantial rise in the yield means that the Class of 2002 is now full, and it will probably be impossible to admit anyone from the waiting list. In more typical years, the College has been able to admit between 50 and 100 from the waiting list.

"We are extremely pleased that the College has again attracted so many extraordinarily talented students this year," said William R. Fitzsimmons '67, Dean of Admissions and Financial Aid. "With many leading American and international universities recently announcing changes in their financial aid programs designed to compete more aggressively for top students, the leadership of Dean of the Faculty of Arts and Sciences Jeremy Knowles and President Neil Rudenstine allowed Harvard to extend its best welcome to prospective members of the Class of 2002."

The Dean and President reemphasized their unwavering commitment to a strong need-based financial aid program and to the policy of admitting the best students without regard to their financial circumstances. With nearly 70 percent of all undergraduates on financial aid, and with scholarship grants of $45 million, Harvard has always been a leader in financial aid.

Dean Knowles stated in February, "We shall set no limit on the financial resources necessary to make Harvard College fully accessible to all students of promise. . . Students who are admitted to next fall's entering class will receive competitively supportive offers, and financial aid will be tailored flexibly and individually."

James S. Miller, director of financial aid, and his staff were available weekdays from 8 a.m. to 8 p.m. and several Saturdays for the month of April, and talked with an unprecedented number of students and parents about their financial aid awards. "Jim and his staff worked extremely hard to make it possible for

EXHIBIT C-7

Early reports

Clinical evidence of angiogenesis after arterial gene transfer of $phVEGF_{165}$ in patient with ischaemic limb

Jeffrey M Isner, Ann Pieczek, Robert Schainfeld, Richard Blair, Laura Haley, Takayuki Asahara, Kenneth Rosenfield, Syed Razvi, Kenneth Walsh, James F Symes

Summary

Background Preclinical findings suggest that intra-arterial gene transfer of a plasmid which encodes for vascular endothelial growth factor (VEGF) can improve blood supply to the ischaemic limb. We have used the method in a patient.

Methods Our patient was the eighth in a dose-ranging series. She was aged 71 with an ischaemic right leg. We administered 2000 μg human plasmid $phVEGF_{165}$ that was applied to the hydrogel polymer coating of an angioplasty balloon. By inflating the balloon, plasmid DNA was transferred to the distal popliteal artery.

Findings Digital subtraction angiography 4 weeks after gene therapy showed an increase in collateral vessels at the knee, mid-tibial, and ankle levels, which persisted at a 12-week view. Intra-arterial doppler-flow studies showed increased resting and maximum flows (by 82% and 72%, respectively). Three spider angiomas developed on the right foot/ankle about a week after gene transfer; one lesion was excised and revealed proliferative endothelium, the other two regressed. The patient developed oedema in her right leg, which was treated successfully.

Interpretation Administration of endothelial cell mitogens promotes angiogenesis in patients with limb ischaemia.

Lancet 1996; **348:** 370–74

Introduction

Among the growth factors that promote angiogenesis, vascular endothelial growth factor (VEGF),[1] also known as vascular permeability factor,[2] and vasculotropin,[3] is specifically mitogenic for endothelial cells. The first exon of the VEGF gene includes a secretory signal sequence that permits the protein to be secreted naturally from intact cells.[3] We have shown[4,5] that arterial gene transfer of naked DNA encoding for secreted protein yielded physiological levels of protein despite low transfection efficiency. Site-specific gene transfer of plasmid DNA encoding the 165-aminoacid isoform of human VEGF ($phVEGF_{165}$) applied to the hydrogel polymer coating of an angioplasty balloon,[6] and delivered percutaneously to the iliac artery of rabbits in which the femoral artery had been excised to cause unilateral hindlimb ischaemia led to development of collateral vessels and increased capillary density, improved calf blood-pressure ratio (ischaemic/normal limb) and increased resting and maximum vasodilator-induced blood flow.[6-10] We now use this strategy in the ischaemic limb of a patient.

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Patient and methods

Patient

A 70-year-old non-diabetic woman was referred for gangrene of the right great toe. About a year earlier, the patient had cramping right-foot pain; several corns were removed, she was given intramuscular cortisone, prescribed ibuprofen, and fitted with shoe inserts. Symptoms worsened and the patient received oxycodone, hydrocodone, and a fentanyl patch. The great toe lesion progressed to gangrene, and the second and third toes became compromised. She had no palpable pedal pulses of the right limb. Ankle-brachial index of the ischaemic limb was 0·26. Arteriography revealed a 40% stenosis of the proximal popliteal artery, and occlusion of the peroneal, anterior tibial, and posterior tibial arteries midway to the foot. Surgical exploration of the distal right limb failed to identify a suitable site for a bypass.

The patient was suitable for arterial gene therapy according to a protocol[11] approved by the Human Institutional Review Board and Institutional Biosafety Committee of our centre, as well as the Recombinant DNA Advisory Committee of the National Institutes of Health and the US Food and Drug Administration.

Plasmid DNA

$phVEGF_{165}$ consists of a eucaryotic PUC 118 expression vector into which cDNA encoding the 165-aminoacid isoform of VEGF has been inserted.[11] A 763 basepair cytomegalovirus promoter/enhancer is used to drive VEGF expression. The PUC 118 vector includes an SV40 polyadenylation sequence, an *Escherichia coli* origin of replication, and the β-lactamase gene for ampicillin resistance. The plasmid was prepared in the Human Gene Therapy Laboratory at our centre from cultures of $phVEGF_{165}$-transformed *E coli*, purified with a Qiagen-tip 2500 column, precipitated with isopropanol, washed with 70% ethanol, and dried on a Speed Vac. The purified plasmid was reconstituted in sterile saline, stored in vials, and pooled for quality control analyses (absorbance at wavelengths of 260 and 280 nm to document ratio between 1·75 and 1·85; limulus amoebocyte lysate gel-clot assay [BioWhittaker] to establish bacterial endotoxin levels below 5 endotoxin units per kg bodyweight; microbial cultures; southern blot for level of contaminating genomic *E coli* DNA; and ethidium bromide staining after agarose-gel electrophoresis to confirm that over 90% of the nucleic acid was in the closed, circular supercoiled form). To confirm the identity of the prepared plasmid, the VEGF-coding region from each pooled batch was resequenced (Applied Biosystem 373A).

Percutanous arterial gene transfer

Arterial gene transfer was done with a hydrogel-coated balloon-angioplasty-catheter (Boston Scientific).[6] A sterile pipette was used to apply 2000 μg plasmid DNA at 10·3 μg/μL in 194·2 μL

EXHIBIT D

DISCLOSURES

Growth factors can be utilized to induce the growth of "hard tissue" or bone and "soft tissues" like ectodermal and mesodermal tissues. As used herein, the term growth factor encompasses compositions and living organisms which promote the growth of hard tissue, such as bone, or soft tissue in the body of a patient. The compositions include organic and inorganic matter. The compositions can be genetically produced or manipulated. The living organisms can be bacteria, viruses, or any other living organism which promote tissue growth. By way of example and not limitation, growth factors can include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (acidic/basic)(FGF a,b), interleukins (IL's), tumor necrosis factor (TNF), transforming growth factor (TGF-B), colony-stimulating factor (CSF), osteopontin (Eta-1 (OPN), platelet-derived growth factor (PDGF), interferon (INF), bone morphogenic protein 1 (BMP-1), and insulin growth factor (IGF). Recombinant and non-recombinant growth factors can be utilized as desired. Bacteria or viruses can, when appropriate, be utilized as growth factors. For example, there is a bacterial hydrophilic polypeptide that self-assembles into a nanometer internal diameter pore to build a selective lipid body. Various enzymes can be utilized for the synthesis of peptides which contain amino acids that control three-dimensional protein structure and growth. Growth factors can be applied in gels or other carriers which regulate the rate of release of the growth factors and help maintain the growth factors, and the carrier, at a desired location in the body. Time release capsules, granules, or other carriers containing growth factor can be activated by tissue pH, by enzymes, by ultrasound, by electricity, by heat, by selected in vivo chemicals or by any other selected means to release the growth factor. The carrier can be resorbable or non-resorbable. Or, the growth factor itself can be activated by similar means. Either the carrier or the growth factor can mimic extracellular fluid to control cell growth, migration, and function. The growth factor can be administered orally, systemically, in a carrier, by hypodermic needle, through the respiratory tract, or by any other desired method. The growth factor can also be administered into a capsule or other man-made composition or structure placed in the body. While administration of the growth factor is presently usually localized in the patient's body, circumstances may arise where it is advantageous to distribute a growth factor throughout the patient's body in uniform or non-uniform concentrations. An advantage to growth factors is that they can often, especially when in capsule form or in some other containment system, be inserted to a desired site in the body by simply making a small incision and inserting the growth factor. The making of such a small incision comprises minor surgery which can often be accomplished on an out-patient basis. The growth factors can be multifactorial and nonspecific.

In another embodiment of the invention, genetically produced living material is used to form an implant in the bone of a patient. The DNA structure of a patient is analyzed from a sample of blood or other material extracted from a patient and a biocompatible tooth bud **122** (FIG. 3) is produced. The bud **122** is placed in an opening **123** in the alveolar bone and packing material is placed around or on top of the bud **122**. The size of opening **123** can vary as desired. The packing around bud **122** can comprise HAC **124**, hydroxyapatite, blood, growth factors, or any other desirable packing material. The bud **122** grows into a full grown tooth in the same manner that tooth buds which are in the jaws of children beneath baby teeth grow into full sized teeth. Instead of bud 122, a quantity of genetically produced living material which causes bud **122** to form in the alveolar bone can be placed at a desired position in the alveolar bone such that bud **122** forms and grows into a full sized tooth. Instead of forming an opening **123**, a needle or other means can be used to simply inject the genetically produced living material into a selected location in the alveolar bone. As would be appreciated by those skilled in the art, genetically produced materials can be inserted in the body to cause the body to grow, reproduce, and replace leg bone, facial bone, and any other desired soft and hard tissue in the body.